Neuroimmune Signaling in the Hippocampus: Mechanisms of Risk and Resilience

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Psychology & Neuroscience in the Graduate School
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ABSTRACT

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Abstract

The interactions between the brain and the immune system are extensive and each has a profound influence on the other. The hippocampus is a brain region that is strongly impacted by the immune system, especially considering its large population of microglia, the resident immune cells of the brain. Cytokines and chemokines, the signaling molecules from immune cells, signal within the central nervous system (CNS) as well, and they are critical in hippocampal function. The relationship between the immune system and the hippocampus may underlie its particular vulnerability to diseases and disorders of the nervous system and the periphery. Conversely, immune signaling within the hippocampus is affected by alterations in hippocampal resilience and flexibility, such that increased hippocampal plasticity reduces vulnerability to immune challenges. The balance between risk and resilience in the hippocampus is modulated by immune signaling, especially by microglia.

The hippocampus is vulnerable to immune challenges, disease and injury, but it is simultaneously a region capable of profound plasticity and flexibility. The following dissertation experiments were designed to assess the roles of microglia and their signaling molecules, cytokines and chemokines, during normal hippocampal processes, such as learning and memory and response to immune challenge. The first set of experiments examined the effects of a neonatal bacterial infection in rats on hippocampal-dependent learning and memory as well as neuronal and microglial

signaling in adulthood. In the first experiment, neonatally infected rats have impaired memory during fear conditioning following an immune challenge in adulthood. The impairment is caused by the exaggerated expression of the pro-inflammatory cytokine, interleukin (IL)-1β, within the hippocampus during learning. Hippocampal microglia are the primary source of IL-1β and the microglia in neonatally infected rats are "primed" by the infection into adulthood. In the second experiment, neonatally infected rats are more accurate on a Morris Water maze task following minimal training in adulthood, but have significantly impaired memory for a reversal platform location. In addition to improved accuracy, they have lower neural activation as measured by Arc protein expression within the dentate gyrus (DG) of the hippocampus. The next set of experiments assessed the effects of increasing hippocampal plasticity on immune signaling within the hippocampus. Following 7 weeks of environmental enrichment (EE), enriched rats had an attenuated pro-inflammatory response within the hippocampus in response to an *in vivo* peripheral immune challenge. The reduced immune response was specific to a subset of cytokines and chemokines and occurred only within the hippocampus and not adjacent cortical regions. Enrichment increased glial antigen expression within the DG as well. In another group of enriched rats, an *ex vivo* stimulation of isolated hippocampal microglia from EE rats demonstrated that the reduced microglial reactivity observed *in vivo* requires influence of other neural cell types on microglia phenotype, such that microglia within the DG of EE rats are smaller

than controls. Taken together, these experiments define cellular and molecular mechanisms of hippocampal vulnerability and resilience as a function of interactions between the brain and the immune system.

Dedication

This work is dedicated to my family. It is dedicated to my paternal grandparents, Betty and Arba Jr. who always supported my scholarly efforts, allowed me to help with crossword puzzles and taught me about nature, animals and the world we live in. It is dedicated to my maternal grandparents, Ruth and Stanley, who still ask me what I am doing at work, attempt to read my latest publications and send me articles from the New York Times about the latest and greatest in mouse and brain research. To have come from a family in which all 4 of my grandparents went to college and both of my grandfathers received advanced degrees is a rare and wonderful blessing that has allowed me to pursue academia.

My work in graduate school is also dedicated to my aunts and uncles – Janet and Paul Bliss, Barbra and Bruce Rohrer, Maggie Leshen and Howard Faigel, and Margot Welch and Alan Harwood. I am so lucky to have three aunts and a great-aunt who are scholars and scientists in their own rights. I am even luckier to have relatives who are willing to listen to my latest harebrained ideas and strange job descriptions without raising too many eyebrows. It's also been a blessing to have fellow academics in the family – your experiences have made succeeding at mine all the easier.

Finally, all of this work is dedicated to my immediate family – my mother Cindy, my father Arba III, and my brother Arba IV (AG). With parents who were a math and economics double major and a chemistry major respectively, it is hard to escape the

"nerd" in our genetics. I am so lucky to have parents who let me do experiments on bubbles at my 8th birthday party and who didn't blink when I said I would be staying for the summer at college to work with rats. To all three of you – I could not have gotten through the past 5 years without your help, support and love. Thank you for not worrying too much about how nerdy I am, and for cheering me on through this whole process.

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Acknowledgements

All of this work could not have been accomplished without the assistance and guidance of many people along the way. I thank Martha Simms Mikell and Gene Arcand for insisting that I take that last year of Physics at HRA, even when I'd finished my requirements. I am deeply grateful to Dr. Noah Sandstrom for drinking tea with me in the Schow Atrium while we talked over my question from class, and then accepting me into his laboratory and training me to be a fledgling scientist. Without his influence and mentorship, I never would have made my way to Duke or to be a neuroscientist.

I am grateful to Dr. Warren Meck and Dr. Christina Williams for hiring me as their lab technician at Duke and mentoring me through my graduate school application process. Warren was incredibly supportive of my efforts to design my own projects and his trust in me was invaluable. The guidance I received from Tina from the first day as her technician to my final semester in graduate school has been one of the major foundations to my success. A fellow Williams alumna and consummate scientist and mentor, she will be an important mentor to me for years to come.

I am thankful for all the members of the Bilbo Lab at Duke as it grew into the bustling place that it is today. Dr. Jaclyn Schwarz was the best office mate a young scientist could have asked for – always willing to give me advice, to let me bounce ideas off of her and to give me guidance in my efforts. She continues to be a mentor and an inspiration, even from afar. I thank Jessica Bolton for being my colleague and friend,

keeping me full of laughter and intensity – your first stay in my house signaled the beginning of a beautiful friendship! I am grateful to Rishi Mistry, Paige Sholar, Christine Belliveau, Melanie Wiley and Stewart Cox for their assistance in all the tasks that make the science world go 'round. I also thank Rishi and Paige most especially for their friendship during the first years of graduate school and for their attempts to preserve my sanity, to support my culinary endeavors and to increase the amount of laughter in my life. I am so pleased to have had Michael Lacagnina as my office mate for the last year of graduate school, keeping our office full of science talk and humor at all hours.

I would like to thank, in no particular order, the other graduate students, post-docs and faculty in several departments who were invaluable for their insight, encouragement and support over the years: Dr. Cagla Eroglu, Dr. William Parker, Dr. Ramona Rodriguez, Dr. Susan Smith, Dr. Sandeep Singh, Dr. Fred Nijhout, Dr. Marsha Penner, Dr. Kristen Pleil, Dr. Richa Hanamsagar, Yuliya Nikolova, Sharda Umanath, Samantha Deffler, Allison Detloff, Nathan Hedrick, and Sydney Koke.

Last, and by no means least, I am thankful for my mentor, Dr. Staci Bilbo. She took a chance on me as her first graduate student, and I have learned so much from her about being a scientist and about being a mentor. The first time I heard her talk about microglia, I was hooked for life. Staci knew when to push me and keep me motivated, and her contagious excitement about new projects and data made it easy to come into lab every day. Her patience and empathy were incredible gifts during the difficult times.

She taught me the importance of collaboration and the invaluable skill of asking for help when I needed it. I look forward to collaborating with her in the future and to knowing that her expertise will be helpful to me for years to come.

1. Introduction

The brain and the immune system are extensively interconnected and each cannot function without the other. Indeed, there is a growing body of evidence that molecules once thought to function exclusively within the peripheral immune system are required for many normal processes within the central nervous system (CNS). The hippocampus is critical for learning and memory, and its unusual structure and signaling properties are thought to underlie its important role in cognition. The hippocampus is incredibly plastic, especially as one of only two robust neurogenic regions in the mature brain. The hippocampus is also remarkably vulnerable to injury, insult, and CNS disorders, such as ischemia and Alzheimer's disease. The experiments in this dissertation provide evidence that immune molecules in the CNS and neuroimmune communication directly connect the unique plasticity and vulnerability of the hippocampus.

Microglia, the primary immunocompetent cells in the CNS, densely populate the hippocampal formation. They can receive inputs from neurotransmitters and hormones, but their most common signaling molecules, cytokines and chemokines, were first defined by their roles in the immune system. As recent studies have shown, cytokines and chemokines are important for many aspects of neural function, and without them, brain homeostasis and cognitive performance suffers. They have roles in learning and memory paradigms (e.g., interleukin-1β in fear conditioning), plasticity (e.g.,

interleukin-6 in LTP maintenance) and neurogenesis (e.g., CXCL12 in GABA signaling with newborn neurons): all critical functions and traits of the hippocampus. There is increasing support for the notion that hippocampal function requires microglia and normal neuroimmune signaling, including data that I will describe here.

1.1 The Hippocampus

The hippocampus is a brain region that has been studied as an individual entity for decades. This unique region encompasses the divide between the developing and adult brains and shares important characteristics with both developmental time periods. Its pseudo-developmental phenotype throughout the lifespan plays a role in its unique capabilities for plasticity and flexibility. In examining hippocampal plasticity, much has been learned about the abilities of the adult brain to respond flexibly to both positive (e.g., learning tasks) and negative (e.g., insult) stimuli as well as general properties of neural communication. The hippocampus is known to be a critical neural substrate for many expressions of learning and memory and without it, many learning paradigms are impossible to perform. In sum, the hippocampus offers a unique, well-studied region for understanding brain function, development and plasticity.

1.1.1 Anatomical Organization

The neuroanatomy of the hippocampus is one source of its appeal as a subject of study (for comprehensive review, see 1). Its main neuronal cells – pyramidal and granule cells – are organized in clear layers and its inputs are well-characterized. It is a

cortical structure and like the neocortex, it has large pyramidal and granule cells that signal with and respond to glutamate and acetylcholine and smaller interneurons that are inhibitory. Nevertheless, the hippocampus is distinct from the neocortex in several important ways. First, the hippocampal formation has a distinct laminar structure and development compared to the six layer cortex. The subdivisions of the hippocampus are well-characterized and each has a distinct role in hippocampal function. Second, the hippocampus has primarily unidirectional inputs rather than the radial connectivity (or crosstalk) that occurs in the rest of the cortex. Third, the hippocampus (specifically the dentate gyrus (DG)) shows a steady level of neurogenesis throughout the lifespan whereas the cortex has limited, if any, generation of new neurons. While the hippocampus has been considered a simpler, more accessible version of the cortex by neuroscientists and neurobiologists of the past, the hippocampus has its own remarkable role in brain function that is separate and distinct from other cortical regions.

The hippocampus receives an incredible number of processed sensory inputs from many neocortical regions. However, hippocampal signaling is primarily unidirectional, unlike the bidirectional communication commonly seen within and between cortical layers. Through the perforant path, neurons in layer II of the entorhinal cortex (EC) project into the DG. The three-layer DG contains primarily granule cells packed tightly together in two linear regions often called blades. These two blades meet

to form the V-shape characteristic of this region and the activation of granule cells in response to EC input is relatively sparse compared to the number of inputs received. The convergence of inputs into the DG and its sparse activation are thought to orthagonalize the inputs and to distinguish them from one another, a phenomenon termed "pattern separation" (2, 3). The DG then projects its axons, called mossy fibers, to the CA3 (*cornu Ammonis 3*) pyramidal cell layer and the population of activated cells in the CA3 is larger compared to the number of DG cells projecting to it. The CA3 along with the CA2 and CA1 regions are included in what is often referred to as the hippocampus proper. The strata of the hippocampus proper include the oriens, the pyramidale, the lucidum, the radiatum, the lacunosum and the molecular, each of which contains populations of pyramidal cells similar to the cortex or signaling axons from one region to another. The CA2 region is distinct from both CA3 and CA1, but its inputs are not well-characterized. Inputs to the CA3 originate mostly from the DG, but the heterogeneous population of CA3 pyramidal cells also has associational communication and receives inputs from the EC. From the CA3, the axons (known as Schaffer collaterals, so named for the neuroscientist who first discovered them) project to the CA1 region. The CA1 region also receives input from the entorhinal cortex, but primarily from layer III. This region is thought to organize its inputs in a linear fashion; a phenomenon sometimes termed "pattern completion" (4, 5). The CA1's projections signal to the subiculum, which then projects to the EC, or directly to layer VI of the EC.

The "loop" structure of the hippocampus permits information to be orthogonally and linearly processed before returning to the neocortex. This particular flow of neuronal signaling with its limited redundancy positions the hippocampus in a distinct place in cortical function and may underlie both its critical role in learning and memory as well as its more vulnerable attributes during insults.

1.1.2 Learning and Memory

The hippocampus has numerous important roles in learning and memory. However, the region is best known for its well-characterized roles in two types of learning and memory that are considered unique to this structure – spatial memory, and incidental or episodic memory. Lesion studies, *in vivo* electrophysiology, and newer methods of measuring neuronal activation in the hippocampus have underscored the necessity of a functional hippocampus for successful performance on many behavioral paradigms testing these two types of memory (e.g., water maze test, contextual fear conditioning, inhibitory avoidance, etc.). The hippocampus is central to the brain's interaction with the surrounding environment and using external cues to successfully navigate the world.

Spatial learning paradigms require a functioning hippocampus. Within the CA1 region, place cells respond to specific locations in an explored environment (6) and rodents are skilled at using environmental cues to reach goals in a variety of tasks. One of the best known paradigms for testing spatial learning and memory is the water maze

task, first developed by Richard Morris (7). The task consists of a round pool filled with opaque water in which an escape platform is submerged. During a spatial learning task with a fixed platform, rats must swim in the pool to locate the platform, which remains in a stable location. They are often given 5 to 10 trials per day over the course of several days to learn the location of the platform. This type of water maze task requires the use of distal cues (within the testing room, but outside the "maze" or pool) for acquisition of the platform location, and this type of learning requires the hippocampus (8). Many other behavioral tasks (e.g., Barnes maze, radial arm maze) test spatial learning and memory as well. Often the distinctions between these types of task are based on the goals presented to the rodents during their performances; some relieve stress, others provide appetitive encouragement, but they all require the hippocampus's continual processing of the outside environment for success.

Incidental episodic learning and memory occur passively and automatically in the hippocampus. The exposure to novelty, whether a new space or new object in the environment, is automatically encoded in the hippocampus for possible retrieval during a second exposure to the novel context or relevant cue. For instance, recent technology has allowed researchers to use immediate early genes (IEGs), whose mRNA transcript's cellular kinetics are well-defined, to label the activation of cells in the hippocampus in a time-dependent manner, based on a rat's exposure to an environment one or more times (9-11). Hippocampal neurons are automatically activated by exposure to a new

environment, and monitoring their activation defines the roles of each hippocampal sub-region (11, 12).

One type of incidental learning is classical conditioning, a behavioral paradigm of one-trial learning that has well-defined underlying neural correlates. Classical conditioning is the learned association between a conditioned stimulus (CS; e.g., tone) and an unconditioned stimulus (US; e.g., shock). Contextual fear conditioning assesses the recognition of the context in which fear learning occurs. The context itself can be the singular CS or coupled with another more discrete CS (e.g., tone). For example, in one version of contextual fear conditioning, rats are placed in a novel environment (e.g., a conditioning apparatus comprised of a Plexiglas box with a house light) for 2 to 3 minutes to experience the context. Then a discrete CS (e.g., 15s tone) occurs followed by an aversive US (e.g., 2s 1.5mA shock from a metal grid floor that is connected to a shock generator). The amygdala mediates the explicit association between the cue presentation (CS) and the US (13, 14). The hippocampus interacts with the amygdala to mediate the association between the context and the US, such that rats that experience an aversive US in a specific context will display fear behavior (e.g. "freezing" or immobility, piloerection, shallow breathing) when placed into the context a second time. Their fear behavior is elicited without a second exposure to the US (15). A greater number of exposures to the conditioning context prior to the US presentation increases both the likelihood and the duration of freezing behavior during the subsequent exposures to the

context (16-18). Hippocampal lesions can cause retrograde or anterograde deficits in context recognition, depending on the timing of their application (19-22). In sum, the hippocampus is critical for recognition of the surrounding environment and the cues within it that provide guidance and direction towards desired goals. Importantly, immune molecules have direct effects on the learning and memory capabilities of the hippocampus.

1.2 CNS-Immune System Interactions

The immune system and the brain have a bi-directional relationship that is often mediated by cytokines and chemokines. The dogma of CNS "immune privilege" is no longer accurate as the interactions between neurons, microglia and the immune system are increasingly well-defined (23-25). Sickness behaviors are an example of one such interaction in which a peripheral immune challenge modulates behavior via the brain. Sickness behaviors are characterized by lethargy, anorexia, and a reduction in social and sexual behaviors (26, 27). Sickness behaviors, such as reduced motivation for food or social interactions, can be elicited by cytokine administration in the periphery and within the CNS (28) and does not require exposure to a pathogen or peripheral immune challenge in the periphery to occur. Nevertheless, administration of a pathogen or a substance that activates the immune system in the periphery causes a dramatic increase in cytokine expression that signals to the brain through the vagus nerve, which increases its signaling to the brain after exposure to interleukin-1β (29). Vagotomy reduces the

effects of peripheral circulating cytokines on sickness behavior (30), and direct administration of cytokines or their antagonists into the brain alter sickness behavior (31, 32). Peripheral immune activation can affect the brain through the humoral response as well, in which the complement system is the first responder (33). The complement cascade promotes phagocytosis and inflammatory responses, which can increase these signals in the brain during blood-brain barrier disruption (33). Sickness behavior, which alters a rat's motivational state via increasing brain cytokine or complement levels indicates that much of the immune system's signaling allows the brain to modulate behavior and assist the immune system in ridding the organism of pathogens. In a region like the hippocampus that is constantly incorporating new cells, rewiring and repairing neural connections, increased inflammation may disrupt or irreparably damage these processes.

1.2.1 Cytokines and Chemokines: Signals of the Immune System

Cytokines and chemokines (**chemo**tactic cyto**kines**) are small cell-signaling molecules whose functions have been primarily defined within the immune system, but also have an important role in the nervous system and the interactions between these two systems. The immune system is a diffuse mediator of homeostasis and host defense to environmental perturbations, and cytokines and chemokines maintain communication among the diverse cell types required for immunological function. In the nervous system, they are secreted by neurons and glial cells and are important for

both inflammatory cascades within the CNS as well as normal cognitive function, though the latter remains less defined. In the periphery, chemokines are critical signaling molecules for the recruitment of leukocytes to sites of insult or injury. Chemokines are highly expressed in disease states, including kidney disease (34), atherosclerosis (35), rheumatoid arthritis, HIV-1 infections and cancer (36). They are important for the normal development of immune cells as well, including T-cells (37), B-cells (38, 39), and the survival and development of hematopoietic progenitors (40-42).

The dysregulation of cytokines is linked to many diseases and inflammatory disorders (e.g., atherosclerosis (43) and rheumatoid arthritis (44, 45)). On the other hand, cytokines are critical for normal functions including skeletal development (46), upregulating myelopoiesis during inflammation (47) and hematopoiesis (48). Their importance in cell migration and differentiation in the periphery (46, 48) indicates overlapping mechanisms for their roles in these processes within the brain, directly connecting the central nervous and immune systems.

1.2.2. Immune Signaling Molecules: Chemokines and Cytokines in Normal Brain Function

1.2.2.1 Cytokines

1.2.2.1.1 Interleukin-1β

Cytokines are a large family of molecules that are best characterized for their roles in immunomodulation and inflammation. Interleukin (IL)-1β is a well-characterized cytokine that is important for proinflammatory signaling in the CNS and

the periphery. IL-1β is one of the major signaling molecules that mediate sickness behaviors. It has been implicated in a wide variety of inflammatory disorders as well (e.g., rheumatoid arthritis (49) and type 2 diabetes (50)). The cytokine is also important for hematopoietic precursor development; it is secreted by these precursors and they are regulated through both autocrine and paracrine signaling (51). Its role in the normal development of peripheral immune cells may offer a clue to its critical role in normal hippocampal function as well. The hippocampus is constantly renewing its population of neurons, and IL-1β may influence the migration and eventual fate of NSCs in the perpetual turnover of DG cells.

The role of IL-1β in normal hippocampal function is well characterized (for comprehensive review, see 52). IL-1β is critical in hippocampal-dependent fear conditioning and memory consolidation (53) as well as the maintenance of LTP (54). I.c.v. administration of IL-1β in rats after water maze training improves memory, while i.c.v. administration of IL-1 receptor antagonist (IL-1ra), a cytokine that antagonizes IL-1β function, results in memory impairment on the same task (55). IL-1 receptor knockout mice have impaired learning in the water maze and fear conditioning and have disrupted LTP (56). During hippocampal-dependent learning, IL-1β mRNA is induced in the hippocampus in wild-type mice, but transgenic mice overexpressing IL-1β have impaired hippocampal-dependent memory in the water maze and during fear conditioning (57). As these data show, the expression of IL-1β is required for normal

brain function. Nevertheless, overexpression or reduced expression of this cytokine impairs normal hippocampal function, such that physiological levels are narrowly defined (58). Transplantation of NSCs into the hippocampus from mouse lines with altered IL-1β signaling, as shown in the following studies, alters LTP and hippocampal-dependent memory impairments. Isolated mice with increased IL-1β expression show weight loss, hippocampal-specific impairment in both fear conditioning and water maze, and reduction of LTP; the impairments in the isolated mice are reversed by transplanting NSCs from IL-1 receptor antagonist transgenic overexpression mice (59). Blocking the effects of IL-1β with IL-1ra within the hippocampus alleviates the negative effects of overexpression of IL-1β following the stress of isolation. In IL-1 receptor KO mice, transplantation of NSCs from WT mice into the hippocampus reverses memory impairments in fear conditioning and water maze and restores LTP function in the hippocampus (60). Modulation of IL-1β signaling to appropriate physiological levels prevents or eliminates any deficits caused by under- or overexpression. As these data indicate, IL-1β has a significant role in hippocampal function as do other cytokines.

1.2.2.1.2 Other Cytokines

Many other cytokines play important roles in regulating peripheral immune function and inflammation. Their role as signaling molecules during an acute immune response provides another mechanism by which they may modulate neuronal signaling during normal brain function. Many of these cytokines have been characterized for their

roles in modulating brain function in addition to their roles in the peripheral immune system. For instance, TNFα is a primary signal in neuronal cell death cascades (61) and increased TNFα is associated with Alzheimer's disease, major depressive disorder and other disorders characterized by neuroinflammation. TNFα has never been implicated in short term or long term potentiation *per se*, but it has a critical role in synaptic scaling, the adjustment of synaptic strength based on alterations in network-wide electrical activity. LTP maintenance may require IL-6, though it is difficult to assess in neural tissue (62), and high frequency stimulation in the hippocampus increases IL-6 mRNA expression (63).

LTP is strongly inhibited by interferon-γ (IFNγ) (64). IFNγ also impairs short term plasticity in the CA1 sub-region of rat hippocampal slices (64), and prevents dendritic, but not axonal, outgrowth from rat neonatal hippocampal neuron cultures and induces retractions of dendrite outgrowth (65). Application of IFNγ to rat hippocampal neuronal cultures during the peak of synaptogenesis reduces spontaneous excitatory post-synaptic currents (EPSCs) and increases spontaneous inhibitory PSCs several weeks later (66). Similar to IL-1β, other cytokines can disrupt neuronal communication when they are present in supra-physiological levels.

Inflammation, characterized by overexpression of several cytokines, can impair neurogenesis or new cell survival in the hippocampus. Endotoxin (e.g., lipopolysaccharide or LPS) injection (i.p.) increases neuroinflammation and inhibits

neurogenesis (67). LPS application to neuronal cultures causes a neuroinflammatory response that is reversed by addition of a non-steroidal anti-inflammatory drug (NSAID) to cultures of hippocampal NSCs. Indomethacin, an NSAID, increases neurogenesis in hippocampal cultures from irradiated adult rats and restores neurogenesis to control levels (67). Adult hippocampal neurogenesis is impaired by TNFα signaling mediated by TNF receptor 1 (TNF-R1) basally and following SE, while TNF-R2 does not (68). Proliferation of NSCs is increased by members of the IL-6 family, including LIF and ciliary neurotrophic factor (CNTF). Conversely, IL-6 itself negatively impacts the differentiation of NSCs (69). Curiously, peripheral immune activation in antigen-induced arthritis in mice transiently increases proliferation of NSCs in the hippocampus, such that increased inflammatory tone in the organism encourages neurogenesis (70). While high levels of cytokines, such as IL-1β and TNFα, can disrupt neurogenesis or encourage aberrant neurogenesis (e.g., during seizure), there may also be a role for "alternatively activated" microglia as described above (71-73). For example, microglia stimulated by IL-4 or low levels of IFNγ *in vitro* promote neurogenesis (74). IFNγ applied directly to NSC cultures increases neural differentiation and neurite outgrowth, while TNFα does not (75). Microglial activation in culture can be shifted, and the distinct profiles of secreted cytokines that result from these shifts in phenotype have differential effects on the fates of NSCs (for reviews, see 76, 77). Taken together, these findings demonstrate an important role for cytokines beyond their well-characterized

role in neuroimmune pathologies. The neurogenic niche of the hippocampus relies on these immune molecules to maintain, encourage and, occasionally, inhibit neurogenesis.

1.2.2.1.3 Chemokines

Chemokines are a subset of cytokines (8-12 kD) that are important for chemotaxis of various cell types, including immune and neural cells. The receptors for these small proteins are G-protein-coupled receptors that have 7 transmembrane domains; the similarity between these receptors results in broad promiscuous binding of the chemokine ligands and a lack of specificity between various ligands and their potential receptors (78). The family of chemokines is large with over 40 ligands and a host of receptors that bind to them. In addition to their roles in immune function, increasing evidence demonstrates roles for chemokines in homeostasis in addition to their roles in pathology.

Many chemokine ligands and receptors are important in normal brain function. For instance, CXCR2 expression in HEK cells increases both the apparent affinity of GluR1 homomers for glutamate and the GluR1 homomer channel open probability (79). Application of CXCL2, a ligand for CXCR2, on Purkinje neurons cultured from the cerebellum increases spontaneous AMPA-type glutamatergic excitatory activity (79). Fractalkine (CX3CL1) reduces AMPA-mediated currents in hippocampal neuron culture preparations, through the interaction of CX3CL1 with its receptor, CX3CR1 (80). Fractalkine also alters EPSCs evoked by electrical stimulation of Schaffer collaterals in

patch-clamped CA1 pyramidal neurons in the rat hippocampus (81). The same preparation reduces AMPA current responses through a G_i-coupled dependent attenuation of intracellular cAMP. In hippocampal development, CXCL12 (SDF-1), in concert with its receptor CXCR4, selectively modulates hippocampal neuron axon outgrowth without altering branching (82). CXCR4 modulates synaptic depression in the cerebellum (83). CXCL12 reduces NR2B subunit expression in NMDA receptors on cultured cortical neurons, and the reduction of this subunit decreases calcium excitotoxicity (84). CXCL12 is also directly involved in GABA-ergic transmission within the DG. The chemokine is stored in vesicles of DG basket cells and co-localized with GABA vesicles that synapse onto immature neurons that are integrated in the granule cell layer (85). In transgenic mice that overexpress CCL2 (monocyte chemotactic protein-1; MCP-1), neuronal responsiveness (as measured by extracellular field potentials in hippocampal slices) was reduced compared to wild-type littermate controls. In the same transgenic mice, short term potentiation was enhanced compared to controls and LTP was not affected by chronic exposure to CCL2 (86). Bath application of CCL2 on hippocampal slices in another study increased ESPC occurrence and quantal content (87). Application of chronic CCL3 (macrophage inflammatory protein (MIP)-1α) on hippocampal slices increases intracellular Ca^{2+} levels as well as NMDA-evoked Ca^{2+} signaling and increased numbers of NMDA receptors on the hippocampal neurons (88). These findings indicate roles for chemokines within the CNS and especially the

hippocampus in addition to their well-known roles as inflammatory molecules. While it is likely that chemokines have a part in neural communication in other brain regions, the singular signaling of the hippocampus is modulated heavily by chemokines. Many of these findings were defined *in vitro* and provide a foundation for future *in vivo* characterization of many chemokines, particularly during learning and memory paradigms. Taken together, these data indicate important relationships between chemokines and neurotransmitter signaling, especially in the hippocampus, in the normal brain.

In addition to involvement in neural transmission, chemokines are vital to the migration and development of neural stem cells (NSCs) in the dentate gyrus of the hippocampus (89, 90). Ischemic insults, seizures, or other inflammatory stimuli increase proliferation of NSCs in the DG (91, 92), but NSCs themselves basally express several chemokine receptors, including CXCR4, the receptor for CXCL12, and CXCR2 (90, 93, 94) and are induced to migrate by CCL2 (95). Given the chemotactic roles for chemokines in the peripheral immune system, their similar effects as chemoattractant signals for NSCs highlight a potential evolutionary conservation of these molecules (96) as well as the importance of neuroimmune communication in the normal brain. NSCs can have their own anti-inflammatory effects, especially when transplanted for the treatment of diseases and disorders, such as ischemia and EAE (97-100). The importance of chemokines in maintaining normal neurogenesis provides evidence for the double-

edge sword of the hippocampal pseudo-developmental state –dysfunction of chemokine signaling can lead to damaging inflammatory cascades, but chemokines, and immune molecules in general, are required for normal hippocampal processes. Microglia, the main source of these immune molecules in the brain, also modulate neurogenesis and other hippocampal phenomena.

1.3 Microglia

Microglia are critical for normal brain function, especially as the primary source of cytokines and chemokines in the CNS. Recent work has illuminated the varied and dynamic role of microglia in the CNS, such as release of glutamate and nitric oxide as well as immune signaling molecules (for comprehensive reviews, see 101, and 102). Microglia constantly survey their surrounding microenvironment with their processes and can rapidly respond to changes in the extracellular milieu (103, 104). Microglia can dynamically shift from one activation state to another, altering morphology and phenotype in reaction to CNS and peripheral events. Interestingly, microglia, as long-lived cells in the CNS, have a "memory" for previous activation states. Microglia can be "primed" or sensitized by insult or injury, and these primed cells are more likely to overreact to a subsequent immune challenge. Primed microglia are also more reactive at baseline, indicating an altered reactive tone in the sensitized brain.

In the healthy brain, microglia directly interact with new neurons to modulate neurogenesis; ramified microglia actively phagocytose apoptotic cells during the

generation of new neurons (105). Microglia make direct contact with synapses and dendritic spines to manipulate synaptic remodeling (106, 107). They eliminate dying neurons and damaged presynaptic boutons as well as provide space into which new post-synaptic structures may expand when they insert new receptors during plasticity events (102, 106, 107). In addition to their direct interactions with neurons, microglia are thought to be the primary *in vivo* source of cytokines and chemokines (73, 108-112), both of which are critical families of molecules for maintaining normal brain function.

1.4 Immune Signaling in the Vulnerable Hippocampus

The hippocampus is uniquely sensitive to disruptions in the environment, especially those that activate the immune system, as seen in many CNS pathologies. These pathologies often have more damaging effects in the hippocampus compared to other brain regions. The unique structure of the hippocampus, its perpetual developmental state, and the role of immune signaling molecules in normal hippocampal function may exacerbate the region's vulnerability to injury and inflammatory disorders, especially those disorders that originate in or directly affect the CNS.

1.4.1. CNS Disorders

Several pathologies specific to the CNS have more damaging effects in the hippocampus compared to other brain regions as well, including Alzheimer's disease, seizures and ischemia. The effects of AD on memory processes and hippocampal

function are well-characterized, and neuroinflammation and dysregulation of immune signaling within this region are often considered hallmarks of AD. In both the human and rodent literatures, AD patients and model organisms consistently show increased cytokine and chemokine levels as well as increased microglial activation and activity (IL-1β and IL-2 in humans, (113); IL-1β in humans, (114), human TNF polymorphisms, (115); reactive microglia in human subjects, (116); TGFβ in mice, (117)). Seizures are another disorder that dramatically affects the hippocampus and its function. Immune challenges early in life increase susceptibility to seizures (118-120) and seizures, in turn, increase immune activation in the hippocampus, the thalamus and the neocortex (IL-1β in mice, (121); IL-1β in rats, (122, 123); CCL2 in mice, (124)). Ischemia and the subsequent reperfusion of blood flow to the brain is particularly damaging to the CA1 region of the hippocampus for several days following the insult (125-127). Ischemic events that disrupt blood flow to the CNS (including those caused by cardiac arrest and other blockages) set off a series of inflammatory events, including the activation of microglia and astrocytes that release a storm of cytokines and chemokines (128, 129). Microglia and peripheral macrophages flock to the site of infarction (130-132). Notably, ischemic insults, seizures or inflammatory stimuli markedly impact the proliferation, migration, differentiation and survival of neural stem cells (NSCs) in the DG (91, 92), suggesting an important role for interactions between immune signaling and NSCs in pathology.

1.4.2. Modeling Risk: Neonatal Infection

1.4.2.1. Effects on Learning and Memory

The developing brain is incredibly plastic, but also vulnerable to perturbations in the organism and its environment (for a review of the role of the immune system and the developing brain, see 133). Examining the effects of prenatal and neonatal events on later life outcomes, a field of study known as "early life programming," provides possible mechanisms for neurological dysfunction, obesity and other detrimental disorders (134). Early life programming underscores the sensitivity of the brain during development to a wide number of disturbances. A growing body of research suggests that immune activation during early life has lasting consequences for the immune system, the CNS, and the communication between these two systems (135). Notably, adult hippocampal function is often especially vulnerable to disruption following immune challenges during critical periods of development (136-140). For instance, maternal immune activation or neonatal immune challenge with a diverse number of pathogens and immune activators (e.g., polyriboinosinic-polyribocytidylic acid (poly(I:C)), *Escherichia coli*, lipopolysaccharide (LPS), human immunodeficiency virus (HIV)-1 and IL-6) similarly disrupts spatial learning in adult male rats, suggesting convergence of immune activation onto common plasticity mechanisms (141-146). In our laboratory, neonatal *Escherichia coli* infection has enduring consequences on microglial function, neuroimmune interactions and hippocampal function. In this model, rat pups are injected subcutaneously on postnatal day 4 (P4) with phosphate-

buffered saline (PBS/controls) or a non-lethal dose of live *E. coli*. Neonatal infection with *E. coli* results in increased *IL-1β* and *CD11b* (a microglial antigen) mRNA expression in rat pup hippocampus within 24HR, but the infection is resolved within 72HR (147). In adulthood, these rats are tested for cognitive deficits, using a modified fear conditioning paradigm known as context pre-exposure. Rats experience multiple brief exposures to the conditioning apparatus on the first day. No other stimuli (US or CS) occur during these exposures. The hallmark of the pre-exposure paradigm is the process of repeated exposure to the conditioning context without any other CS or US (15, 16). Forming a representation of the context independent of other discrete cues (e.g. tone or light) requires the hippocampus (20-22). Twenty-four hours later, rats are transported to the conditioning apparatus, where they immediately receive a 2s footshock (US), and are returned to their home cages. Fear behavior is assessed 24HR after the immediate shock. Neonatally infected rats display cognitive deficits in adulthood, but only if they receive an immune challenge of lipopolysaccharide (LPS; 25μg/kg) by intraperitoneal (i.p.) injection after learning (148). Neonatally infected rats that receive LPS immediately following the final episode of context pre-exposure on day 1 have impaired memory for the context 24HR after the shock (48HR after the LPS injection) (148). Importantly, the effects of neonatal infection on adult cognition are specific to the hippocampus. Rats treated with *E. coli* on P4 are not different from controls on hippocampal-independent measures (e.g., freezing to a tone previously paired with shock) (149).

Peripheral immune challenges alter the expression of cytokines and chemokines in the brain (30-32, 108, 147, 148, 150-153). IL-1β is a cytokine that is critical for learning and memory, as described above, and is a potential molecular mechanism for the cognitive impairments observed in the neonatally infected rats. The memory impairment shown in neonatal *E .coli*-treated rats injected with LPS is blocked by intra-cisterna magna injection (i.c.m.) of a caspase-1 inhibitor given prior the LPS injection and subsequent context exposure. Caspase-1 is an enzyme that cleaves the pro-form of IL-1β into its mature active form. Inhibiting caspase-1 prevents the cleavage and the secretion of mature IL-1β protein. Caspase-1 inhibition prior to fear conditioning and LPS administration prevents the hippocampal-dependent learning impairments, underscoring the importance of IL-1β in hippocampal-dependent learning. Inhibiting IL-1β with i.c.m. injection and rescuing cognitive impairments demonstrates that an exaggerated level of IL-1β *within the CNS* in neonatally infected rats impairs cognition (147).

IL-1β is necessary for normal learning and memory (56, 57, 154); therefore its overexpression during cognitive impairments was of particular interest. IL-1β is expressed by neurons, astrocytes and microglia within the CNS, and its source during the observed cognitive impairments in the neonatally infected rats could not be identified from RNA expression and protein concentration data in whole hippocampal tissue. An exaggerated hippocampal IL-1β response to LPS causes impaired memory in

neonatally infected rats, even when the LPS challenge is given 24HR prior to learning (108). In this case, the changes in IL-1β levels must stem from enduring signal modulation by a group or groups of resident cells. Several CNS cell types could be the source of the exaggerated IL-1β; however, the long-lived population of microglia is the primary source of many cytokines and chemokines (58, 109-112, 155, 156) and important for normal brain function (102-104, 106). These microglial cells are reactive to, or "primed" by, previous immune experience and may be permanently altered by prior insult or injury. Thus, it is both possible and likely that the source of exaggerated IL-1β is microglial cells and my work, described subsequently, examines this possibility.

1.4.2.2 Effects on the Neurogenic Niche of the Hippocampus

The development of neurons in the sub-regions of the hippocampus is well-characterized and highly stereotyped. Microglia, however, have a unique developmental trajectory compared to other resident CNS cell types. Microglia do not originate from the neuroepithelium during fetal brain development, but instead colonize the fetal brain from the embryonic yolk sac arising from primitive myeloid progenitors (133, 157-161). They continue their proliferation and development within the CNS into the neonatal period as well (162, 163). A recent study in our laboratory revealed changes in microglial morphology and number throughout ontogeny, from the fetal period to adulthood by labeling the cells with ionized calcium binding adaptor molecule (Iba)1, a constitutively expressed microglial antigen. In male rats, the fetal brain (E17), newborn brain (P0), and

neonatal brain (P4) have greater numbers of Iba1-positive amoeboid microglia and microglia with stout (short, round) processes within the hippocampus. While the morphology of microglia does not directly map onto activation states (73, 164), a population of microglia that consists of amoeboid and stout processed cells, rather than cells with longer thinner processes and smaller cell bodies, likely indicates a more reactive or "primed" population of microglia in the early development of the brain. Later in life, just prior to adolescence (P30) and in adulthood (P60), microglia are more ramified or "quiescent" with significantly fewer cells in the population showing amoeboid or short process morphologies. The distinct development and introduction of microglia into the CNS may underlie the enduring effects of neonatal infection. If microglia are potentially more reactive in the fetal and early neonate brain, they may be more susceptible to immune disruption. The hippocampus, and especially the dentate gyrus, is simultaneously developing during this period of microglial colonization, and immune challenges could be especially detrimental to hippocampal development. *E. coli* exposure affects the neurogenic niches of the hippocampus, such that neonatally infected rats have more activated hippocampal microglia and reduced proliferation of neurons and astrocytes in the hippocampus at P4 compared to controls. In adulthood, *E. coli*-treated rats have decreased neuronal proliferation and decreased undifferentiated cell proliferation compared to controls (165). The enduring changes in microglia occur in parallel to enduring changes in neurogenesis and, potentially, hippocampal plasticity.

1.5 Resilience

As the above sections have shown, neuroimmune interactions influence the susceptibility of the hippocampus to disruptive influences, as well as modulate its functioning in the normal brain. In turn, the hippocampus also has a remarkable capacity for repair and flexibility. This region has a well-documented capability for long-term change following positive interventions (e.g., exercise, environmental enrichment), and that capability for plasticity contributes to its resilience. As a neurogenic region, new cells are continuously added to the hippocampal network and the rate of NSC proliferation and integration in the DG can be modulated by these positive interventions, creating the potential for resilience in the face of future disruptions.

1.5.1. Plasticity

The hippocampus has been a critical structure for characterizing many of the neurobiological mechanisms that underlie neuronal signaling, function and plasticity (for historical overview, see 1). While the hippocampal formation is dramatically different from the adjacent neocortex, its accessibility and large uniform populations of neurons provide an exciting source for neurobiological discoveries. These findings indicate possible cellular and molecular mechanisms for learning and memory, such as plasticity at the neural network, single neuron or even synapse levels in the adult brain (e.g., long-term potentiation (LTP) or AMPA receptor trafficking) (166, 167).

1.5.1.1. Cellular Plasticity

Long-term potentiation (LTP), a phenomenon that strengthens and prolongs connections between neurons, was first observed in early experiments that stimulated the perforant path from the EC to the DG in anesthetized rabbits (168) and, later, in rats (169). High frequency stimulation of this pathway increased excitatory responses from the granule cells of the DG for several minutes following the original stimulation. In the hippocampus, this phenomenon is thought to be mediated by calcium ion (Ca^{+2}) signaling through *N*-methyl-D-aspartic acid (NMDA) receptors in the DG and the CA1 (170, 171). The NR2 subunits of the NMDA receptor have been implicated in LTP (and long-term depression/LTD) (172, 173), but their complete role has yet to be fully characterized. Increasing intracellular Ca^{+2} levels through NMDA receptors leads to the phosphorylation of and trafficking of AMPA receptors to synapses. Altering AMPA receptor number changes the effects of glutamate on the post-synaptic neuron and is thought to underlie the strengthened connections observed during LTP (for review, see 174). To maintain overall structural signaling integrity, there is evidence of a phenomenon called synaptic scaling in which the increase in synaptic strength is spread among all the cells in a network, such that the mean rate of activity is constant and individual cells are not disproportionately strengthened or weakened by LTP (175-177). Dendritic spines that contain AMPA and NMDA receptors are also thought to be formed *de novo* during the induction of LTP (178, 179). Taken together, these data demonstrate

that the study of signaling within the hippocampus provide a foundation for neuronal behavior throughout the brain as well as many possible mechanisms for the functions of the unique structure.

1.5.1.2. Neurogenesis

The plasticity of the hippocampus extends beyond changes on individual neurons to structural alterations as well. Long after the brain is fully developed, the DG of the hippocampus continues to produce and integrate new neurons. Indeed, the DG develops differently from other hippocampal sub-regions, which have their own distinct schedules. The pyramidal neurons of the CA1 and CA3 regions migrate along the ventricle after cell division. CA3 neurons, whose generation peaks on embryonic day 17 (E17) in rats, are generated slightly earlier than CA1 cells, which peak on E18 and 19 (180-182). The DG, on the other hand, begins its development at the same time as CA1 and CA3, but the generation of cells in the DG extends into the neonatal period, and beyond, into adulthood. The majority of DG granule cells are born during the first few weeks of life after birth (183-185). Importantly, the generation of DG granule cells does not cease after the developmental period, but continues into adulthood, albeit at a reduced level. In addition to differentiating into granule cells, the neural stem cells in the subgranular zone (SGZ), a neurogenic cell layer between the granule cell layer and the hilus, can also become astrocytes in the constant turnover of the DG. The capability of the DG to produce and incorporate new cells throughout the lifespan maintains a state

of pseudo-development in the hippocampus. This state offers exciting potential for flexibility and plasticity in hippocampal function, but may contribute to the selective vulnerability of the hippocampus.

In the adult rat, neurogenesis occurs at a remarkable rate in the DG. In early adulthood, rats produce thousands of new cells in the DG daily (186). Few of the new cells survive; however, once new cells differentiate into neurons, they are integrated into the blades of the DG and begin to extend processes and connections to receive inputs as well as extend axonal projections to the CA3 (187, 188). Fully mature adult-born granule cells are functionally and morphologically identical to granule cells born during development (189-191) and are thought to replace a population of dying cells from the developmental period (192). New neurons in adulthood, like those cells in the developing brain, go through distinct morphological (for review, see 193) and excitatory stages. Once they are post-mitotic, these new neurons become excitable first by γ-aminobutyric acid (GABA) and then, as they continue to mature, their excitatory inputs switch to glutamate (190, 191). The purpose of new neurons and their importance for hippocampal function continues to be debated (2, 189, 194-201) and will not be discussed in detail here. Regardless of their function, the consistent turnover of the granule cell population establishes a constantly changing signaling pathway in the hippocampus, especially as the flow of information often passes through the DG on its way to the rest

of the structure. The dynamism of this structure may be the basis for its occasionally fragile hold on normal function and homeostasis.

1.5.2. Modeling Resilience: Environmental Enrichment

Manipulations that positively influence hippocampal function can have enduring effects, even if the manipulations are performed in adulthood. Environmental enrichment (EE) is a housing manipulation that increases physical and social stimuli and has been shown to have profound impacts on hippocampal plasticity and function (202, 203) and on the rest of the brain (204-206). Hippocampal neurogenesis is increased by EE in aged mice, concomitant with decreased anxiety and improved water maze acquisition (207). Cell survival and neurogenesis in the hippocampus are upregulated specifically by running and EE in adult mice (208). Gliogenesis of both astrocytes and microglia is increased in cingulate, motor and visual cortical regions by exposure to a running wheel (209) and astrogliogenesis is upregulated in the dentate gyrus by running (210). Astrocyte morphology is altered in the DG by EE, such that astrocytes differentially extend their processes in parallel with neuronal axon fibers (211). EE increases the expression of hippocampal growth factors (e.g., nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)) (212). BDNF is required for EE-mediated hippocampal neurogenesis as shown in BDNF knockout mice (213). Glial-derived neurotrophic factor (GDNF) is selectively upregulated in the granule cell layer of the DG

and the CA1 of the hippocampus following EE (214). Growth factors are known to increase neurogenesis (215) and modulate synaptic plasticity (for review, see 216).

EE is neuroprotective after insult or injury (e.g., ischemia (217) or seizures (210)). EE rescues cognitive deficits in mice with impaired IL-1β signaling (154). Environmental interventions can also improve outcomes in chronic inflammatory conditions. In a mouse model of AD, running wheel exercise increases clearance of soluble fibrillary Aβ, an especially cytotoxic form of Aβ protein, and decreases inflammatory cytokines (e.g., IL-1β, TNFα) (218). Following whole brain irradiation, growth factor expression, DG neurogenesis and cognitive deficits were rescued by voluntary wheel running, such that mice that ran after irradiation showed an attenuated reduction in neurogenic growth factors, new cells in the DG and cognitive impairments on the Barnes Maze compared to their sedentary counterparts (219). Absent any peripheral immune challenge, wheel running altered cytokine tone in the hippocampi of female mice, reducing TNFα expression and increasing IL-6 and IL-1ra expression (220). Taken together, these data demonstrate that EE is a methodologically simple modulation that augments hippocampal plasticity and that rescues damaged brains from insult or injury. However, the ability of EE to alter neuroimmune function in a potentially neuroprotective manner is relatively unexplored.

1.6 Summary

The following dissertation experiments attempt to analyze the roles of microglia and immune signaling molecules in the seemingly distinct traits of vulnerability, or risk, and plasticity, or resilience. These experiments aim to elucidate the connections between risk and resilience and to demonstrate that they share many of the same molecular mechanisms related to immune signaling within the CNS.

The first set of experiments uses a model of neonatal infection to increase the vulnerability of the hippocampus and to investigate the effects on immune signaling as well as neural signaling within the hippocampus during learning and memory. Previous work with this model has indicated that observed learning impairments in neonatally infected rats are related to signaling within the CNS and enduring, well into old age (147, 165, 221). Two hippocampal-dependent learning and memory paradigms – contextual fear conditioning and the Morris Water Maze task – are used to assess the effects of neonatal infection on learning. The fear conditioning experiments aim to elucidate the cellular and molecular basis for previously observed learning impairments in adulthood that result from neonatal infection, while the Morris Water Maze experiments aim to investigate the effects of neonatal infection on behaviorally complicated tasks and the resulting neural activation within the hippocampus. In sum, these experiments consider the long-term effects of a disruption in immune signaling that occurs early in life.

The second set of experiments was designed to analyze the effects of a positive intervention, environmental enrichment, on immune signaling within the hippocampus. Environmental enrichment has not been used prior to immune challenge before these studies and its effects as a preventative intervention are as yet unknown. In addition to effects on mRNA and protein expression within the hippocampus and nearby regions, these experiments explore the changes that occur specifically within microglia following enrichment. While the first set of experiments in this dissertation examine the effects of altered immune signaling within the CNS on hippocampal function, the second set of experiments addresses the effects of altering hippocampal plasticity on immune signaling within the CNS, as we hypothesize that these traits exist in balance within the hippocampus to maintain the region's unique phenotype.

Research for all experiments was conducted with the approval of the Duke University Institutional Animal Care and Use Committee and the treatment of animal subjects was in accordance with the ethical and human treatment standards of the National Institutes of Health (NIH) and the National Science Foundation (NSF).

2. Microglia and Memory: Modulation by Early-Life Infection

2.1 Introduction

Previous work, as discussed in detail above, demonstrated that neonatal bacterial infection in rats leads to marked HP-dependent memory deficits in adulthood. However, deficits are only observed if unmasked by a subsequent immune challenge (peripheral LPS) 24 h before learning or immediately after learning. These data suggest the infection induces a long-term change within the immune system that, upon activation with the "second hit," LPS, acutely impacts the neural processes underlying memory (147, 148, 222, 223). Indeed, preventing the synthesis of brain IL-1β before the LPS challenge completely prevents the memory impairment in neonatally infected (NI) rats (147).

Earlier experiments, however, did not identify the cellular source of IL-1β during normal learning or possible mechanisms by which this cytokine is enduringly altered by early-life infection. Microglia are the primary immune cells of the brain, are long-lived, and have been linked to neurodegenerative disorders like AD (224, 225). However, whether microglia and their inflammatory products are a cause or consequence of neural dysfunction remains subject to intense debate (225). In this experiment, we assessed microglia as a possible cellular source of IL-1β as well as potential changes in microglial reactivity as a result of neonatal infection. We demonstrate that CD11b+ enriched cells (microglia/macrophages) are functionally primed within the adult HP of

NI rats basally, and produce exaggerated IL-1β *ex vivo*. However, an exaggerated IL-1β response within the HP microglia of NI rats *in vivo* requires LPS before learning. Furthermore, preventing microglial activation during learning prevents memory impairment in NI rats, even if preceded by LPS. Together, these data directly implicate microglial-derived IL-1β in normal learning for the first time. Importantly, they suggest an individual's risk or resilience to neuroinflammatory disorders may critically depend on their early life experience, which can modulate normal cognition- dependent cytokine activity within the brain long after the initial insult.

2.2 Materials and Methods

2.2.1 Animals

Adult male and female Sprague Dawley rats (70 d) were obtained from Harlan and housed in same –sex pairs in individually ventilated, microisolator polypropylene cages, with *ad libitum* access to food and filtered water. The colony was maintained at 22°C on a 12 h light/dark cycle (lights on at 7:00 A.M.). Following acclimation to laboratory conditions, males and females were paired into breeders. Sentinel animals were housed in the colony room and screened periodically for the presence of common rodent diseases; all screens were negative. All experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

2.2.2 Neonatal manipulations

2.2.2.1 Bacterial culture

Escherichia coli culture (ATCC 15746; American Type Culture Collection) vial contents were hydrated and grown overnight in 30 ml of brain-heart infusion (BHI) (Difco) at 37°C. Cultures were aliquoted into 1 ml stock vials supplemented with 10% glycerol and frozen at –20°C. One day before injections, a stock culture was thawed and incubated overnight in 40 ml of BHI at 37°C. The number of bacteria in cultures was read using a microplate reader (Bio-Tek Instruments) and quantified by extrapolating from previously determined growth curves. Cultures were centrifuged for 15 min at 4000 rpm, the supernatants were discarded, and the bacteria were re-suspended in the dose-appropriate volume of sterile Dulbecco's PBS (Invitrogen).

2.2.2.2 Injections

Female breeders were visually examined daily for confirmation of pregnancy, and male breeders were removed from cages before the birth of pups [postnatal day 0 (P0)]. Litters were culled on P4 to a maximum of 10 pups/litter, retaining 2 females and as many male pups as possible. Male pups were injected subcutaneously (30G needle) on P4 with either 0.1 X 10^6 colony forming units of live bacterial *E. coli*/gram suspended in 0.1 ml of PBS ("NI rats"), or 0.1 ml of PBS ("control rats"). This dose of *E. coli* results in sustained (~48-72 h) increased plasma cytokines (IL-1β, IL-6) and corticosterone, as well as specific increases in IL-1β mRNA and related genes (IL-1 type 1 receptor, caspase 1)

within the brain by 24 h after infection (147, 226). All pups were removed from the mother at the same time and placed into a clean cage with bedding, weighed and injected individually, and returned to the mother as a group. Elapsed time away from the mother was <5 min. All pups from a single litter received the same treatment due to concerns over possible cross-contamination from *E. coli*. All injections were given between 2:00 P.M. and 4:00 P.M. All male pups for adult analyses were weaned on P21 and housed in sibling pairs; remaining female pups were killed. To control for litter effects, a maximum of two pups per litter across a minimum of three different litters were assigned to a single experimental group. All rats were tested as adults between 65 and 90 d of age.

2.2.3 Behavioral Procedures

2.2.3.1 Contextual and auditory cue fear conditioning

2.2.3.1.1 Apparatus

The conditioning context consisted of one of two identical black Plexiglas boxes with open fronts [41.9 cm length (L) X 40.6 cm width (W) X 46.2 cm height (H)] to allow viewing of animals. The conditioning chambers [30.5 cm (L) X 26.7 cm (W) X 33 cm (H)], within each black box, are made of clear Plexiglas with metal side walls (Coulbourn Instruments). Each chamber has a ceiling-mounted infrared activity monitor connected to a PC. A phasic auditory cue (2976 Hz tone presented at 76 dB) is also mounted on a ceiling of each black box. A 2 s, 1.5 mA shock is delivered through a removable floor of

stainless-steel rods. Each rod is 0.5 cm in diameter, is spaced 1.75 cm center to center, and is wired to a shock generator and scrambler. The chamber was cleaned with 70% alcohol and then water before each animal.

2.2.3.1.2 Procedure

Rats were allowed to explore the chamber for 2 min before the onset of a 15 s tone, followed immediately by a 2 s footshock (1.5 mA). Immediately after the termination of the shock, rats were removed from the chamber. Rats were tested 1 or 72 h later for contextual fear in the original conditioning chamber for 6 min. For assessment of auditory-cue fear (3 h after 72 h context test), testing occurred in a novel chamber with triangular Plexiglas walls. The rod floors were removed, and a 7 W, 120 V AC clear, red light bulb illuminated the chests. Freezing was again assessed for 6 min. The auditory conditioned stimulus (CS) was absent for the first 3 min (pre-CS) and present for the last 3 min (CS period). Freezing represents rats' dominant defensive fear response and is characterized by an immediate suppression of behavior accompanied by immobility, shallow breathing, and a variety of other autonomic changes, including an increase in heart rate and piloerection. Freezing in these experiments was defined as the absence of all visible movement, except for respiration. Scoring began 10 s after the animal was placed into the chamber.

2.2.3.2 Open field

To assess overall activity levels in unmanipulated rats treated with water versus minocycline (MINO) (50 mg/kg 12 h prior plus 25 mg/kg 1 h prior via oral gavage), rats were placed into a novel open field [45.1 cm (W) X 40.0 cm (L) X 34.9 cm (H)] beginning 1 h after the final dose, and scored for 10 min using automated tracking software (ANYmaze; Stoelting).

2.2.4 Drugs

LPS derived from *E. coli* (serotype 0111:B4; Sigma-Aldrich) was suspended in sterile saline and administered via intraperitoneal injection at a dose of 25 μg/kg and a volume of 1 ml/kg. This dose was selected based on our previous body of work (147-149, 165, 221-223, 227, 228). Minocycline hydrochloride (Sigma-Aldrich) was dissolved in water to a dose of 50 or 25 mg/kg in a volume of 5 ml/kg and administered via oral gavage (=10 and 5 mg/ml, respectively) using animal feeding needles (Harvard Apparatus; catalog #52-4199). The average body weight of the adult rats was 400 g. Rats were acclimated to the procedure using water for 3 d before each experiment.

2.2.5 Tissue collection

Adult rats were deeply anesthetized with a ketamine/xylazine mixture and transcardially perfused with ice-cold saline for 2 min to clear brain vessels of blood and peripheral cells. Brains were extracted and immediately dissected into prefrontal cortex (PFC), HP, and adjacent parietal cortex (PCX), depending on experiment, on an ice-

chilled plate. Each reach was cut in one half along the sagittal plane, and one-half regions to be used for mRNA and protein analysis were placed into separate RNase- and endotoxin-free microcentrifuge tubes and snap frozen in liquid nitrogen. Tissues were stored at –80°C until processing. Brain hemispheres collected for assessment of protein versus mRNA were alternated between animals and distributed equally across groups.

2.2.6 IL-1β ELISA

Brain tissue samples were sonicated in cold Iscove's culture medium containing 5% fetal calf serum and a mixture enzyme inhibitor (in mM: 100 amino-*n*-caproic acid, 10 EDTA, 5 benzamidine-HCl, and 0.2 phenylmethylsulfonyl fluoride), and IL-1β was measured from the supernatant using a commercially available ELISA kit (R&D Systems). The detection limit for IL-1β was 5 pg/ml. To adjust for variability in tissue sample sizes, total protein concentrations were determined by Coomassie Brilliant Blue AG-250 protein assay (180). Cytokine data are expressed as pictograms per 100 μg of total protein.

2.2.7 Real-time qPCR

For whole tissues, total RNA was isolated based on the TRIzol method (229). Following RNA isolation and enrichment, samples were DNase-treated, and cDNA was synthesized using the reverse transcriptase kit from QIAGEN. Amplification of cDNA was performed using a QuantiFast SYBR Green PCR kit (QIAGEN) on a Mastercycler ep *realplex* (Eppendorf). cDNA (1.5 μg in 1 μl) was added to a reaction master mix (12 μl)

containing HotStarTaq Plus DNA Polymerase, QuantiFast Buffer, SYBR Green I, Passive reference dye, and gene-specific primers (500 nM each of forward and reverse primer). Threshold cycle (C_T) (number of cycles to reach threshold of detection) was determined for each reaction, and relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (230, 231) as follows: (1) we normalized the cycle threshold (C_T) of the target gene of interest (T) to that of the reference (housekeeping) gene (R), for both the test (A) and calibrator samples (B): $\Delta C_{T(A)} = C_{T(T,A)} - C_{T(R,A)}$ and $\Delta C_{T(B)} = C_{T(T,B)} - C_{T(R,B)}$. We defined the calibrator samples as that with the lowest (baseline) expression. (2) We normalized the ΔC_T of A to B: $\Delta\Delta C_T = \Delta C_{T(A)} - \Delta C_{T(B)}$, and (3) we calculated the expression ratio: $2^{-\Delta\Delta Ct}$ = normalized expression ratio.

2.2.7.1 Primer specifications

Primer pairs for CD200 and CD200R were purchased from QIAGEN. We designed primers for CD11b, glial fibrillary acidic protein (GFAP), IL-1β, CX3CL1, CX3CR1, GAPDH, and 18s: cDNA sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and designed using an online Oligo Analysis and Plotting Tool (QIAGEN), which were tested for sequence specificity using the basic local alignment search tool at NCBI. Primers were obtained from Sigma-Aldrich, and specificity was verified by melt curve analysis. We estimated the efficiency of each primer set based on serial dilution standard

curves. All primer sets were estimated as >90% efficient, and within 5% of each other. Primer sequences are displayed in Table 1.

2.2.8 Microglial isolation

Isolated HPs were sliced into small pieces using a sterile razor and transferred into a 2 ml sterile tube with 1 ml of HBSS (w/o) (Invitrogen), which was spun at 300 X *g* at room temperature for 2 min. HP tissue was then brought to a single-cell suspension using Miltenyi's Neural Tissue Dissociation Kit (P) (Miltenyi Biotec) in MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 7.2) for 15 min at 4°C. HP tissue was then washed with 5 ml of MACS buffer and centrifuged at 300 X *g* at 4°C for 10 min. The tissue was resuspended in 500 µl of MACS buffer and passed through a nylon filter on LD columns (Miltenyi Biotec) exposed to a strong magnetic field. The flow through (demyelinated HP cells) and subsequent washes were collected into a new 15 ml conical and centrifuged at 300 X *g* at 4°C for 10 min. The demyelinated HP cells were immediately taken for flow-cytometric analysis (see Figure 5) or further fractionated based on CD11b expression as follows.

2.2.8.1 MACS enrichment of CD11b+ cells

Cells were resuspended in 500 µl of MACS buffer, stained with 5 µl of PE-conjugated mouse anti-rat CD11b/c (BD Biosciences Pharmingen) and incubated for 10 min in the dark at 4°C. The cells were washed with 5 ml of MACS buffer and centrifuged at 300 X *g* at 4°C for 10 min. Cells were then incubated with anti-PE MicroBeads

(Miltenyi Biotec) at 4°C for 15 min. The suspension was washed with 5 ml of MACS buffer and centrifuged at 300 X *g* at 4°C for 10 min, and the supernatant was aspirated off. The cells were resuspended in 500 µl of MACS buffer and were passed through a nylon mesh filter onto magnetized LS columns (Miltenyi Biotec). The columns were washed with 4 ml of MACS buffer and the flow through either collected into a clean 15 ml conical (as CD11b/c-negative fraction) or discarded. Magnetically labeled CD11b/c-positive cells were flushed out of the columns twice with MACS buffer into a clean 15 ml conical. Magnetically enriched cell fractions were centrifuged at 300 X *g* at 4°C for 10 min, followed by resuspension in 500 µl of PBS (Invitrogen) or Neuro Media (high-glucose DMEM supplemented with 20% FBS and 5 µg/ml forskolin). Isolated CD11b+ and CD11b- cells were then taken for flow-cytometric analysis (see Figure 6a), immediately lysed for RT-PCR analysis (see Figure 7), or placed into culture as follows.

2.2.8.2 *Ex vivo* LPS stimulation

Isolated CD11b+ or CD11b- cells were suspended in high-glucose DMEM plus 20% FBS and 5 µg/ml forskolin and plated in 96-well round-bottom plates at a density of 2 X 10^5 cells/200 µl/well. Cells were incubated with LPS (10 ng/ml) or media alone for 4 h at 37°C, 5% CO_2. For each condition, samples were run in duplicate. At the end of incubation, the plate was centrifuged at 1000 X *g* for 10 min at 4°C to pellet cells. For RNA isolation, medium was removed and cDNA synthesized using the SuperScript III CellsDirect cDNA synthesis system (Invitrogen), according to the manufacturer's

instructions. Real-time qPCR was performed as described above, and the level of IL-1β mRNA was quantified relative to the housekeeping gene 18s.

2.2.9 Fluorescence staining and flow cytometry

Demyelinated or MACS-enriched HP cells were washed once in PBS supplemented with 0.5% BSA and 2 mM EDTA (MACS buffer), followed by centrifugation at 350 X *g* for 5 min. MACS buffer was removed by decanting or aspiration and the pellet dispersed with agitation. Cells were incubated with 5 µl of rat Fc receptor block (CD32; BD Biosciences Pharmingen) for 5 min at 4°C. Next, 100 µl of PE-conjugated mouse anti-rat CD11b/c (BD Biosciences Pharmingen), diluted 1:500 in MACS buffer, was added, and cells were incubated for 15 min at 4°C in the dark. Because CD11b/c labels all macrophages, CD11b+ in a control experiment were incubated in anti-CD11b/c-PE and anti-CD45-FITC (BD Biosciences Pharmingen) to determine the relative concentration of perivascular macrophages, which label as CD11b+ / CD45 ^{high} (232). Cells were washed in MACS buffer, spun down at 350 X *g* for 5 min, and t hen fixed in 200 µl of 1.5% paraformaldehyde before analysis using a FACS Canto II flow cytometer (BD Biosciences) and FlowJo software (Tree Star). For each sample, 10,000 events were collected and doublets were excluded from the analysis based on properties of size (forward scatter height and area).

2.2.10 Data analysis and statistics

Data were analyzed using SigmaStat statistical software. For significant *F* scores, Holm-Sidak *post hoc* comparisons were performed to further distinguish among groups, and all differences were considered statistically significant if $p < 0.05$.

2.3 Results

2.3.1 Learning increases IL-1β protein specifically within the hippocampus, and this increase is modulated by neonatal infection

We have reported that systemic infection with *E. coli* on P4 leads to marked HP-dependent memory impairment in adulthood, but only if these animals receive a low-dose LPS challenge after learning. The impairment is causally linked to exaggerated CNS IL-1β production, as preventing IL-1β synthesis within the brain completely prevents the memory impairment (147). Peripheral cytokine levels do not differ between groups in response to LPS, suggesting the critical difference within the brain is not due to a differential signal from the periphery (147). Curiously, LPS 24 h prior to learning also impairs long-term contextual memory in NI but not control rats despite the fact that HP IL-1β is largely undetectable in both groups 24 h after an LPS injection alone (147, 149). These data led us to test the hypothesis that learning itself induces IL-1β protein within the HP, which is differentially modulated in NI rats. To test this, rats treated with PBS or *E. coli* on P4 were injected intraperitoneally with saline (SAL) or LPS as adults, and fear conditioned 24 h later ($n = 8$/group) (Figure 1a). Rats were assessed for memory 72 h after fear conditioning (Figure 1c). Figure 1*B* illustrates that freezing to the context

did not differ by neonatal treatment group at the short-term (1 h) memory test. Interestingly, there was a main effect of injection ($F_{(1, 31)} = 1.27$; $p = 0.001$). *Post hoc* tests revealed that freezing was significantly lowest in the NI plus adult LPS group. In contrast to contextual memory, which depends on the HP, freezing to the tone did not differ by neonatal group in LPS-injected rats at the 72 h test, either before the tone presentation (pre-CS) or during the tone (CS) (Figure 1d). As expected, freezing was significantly greater to the CS than during the pre-CS period in all rats ($F_{(1, 31)} = 61.6$; $p = 0.001$).

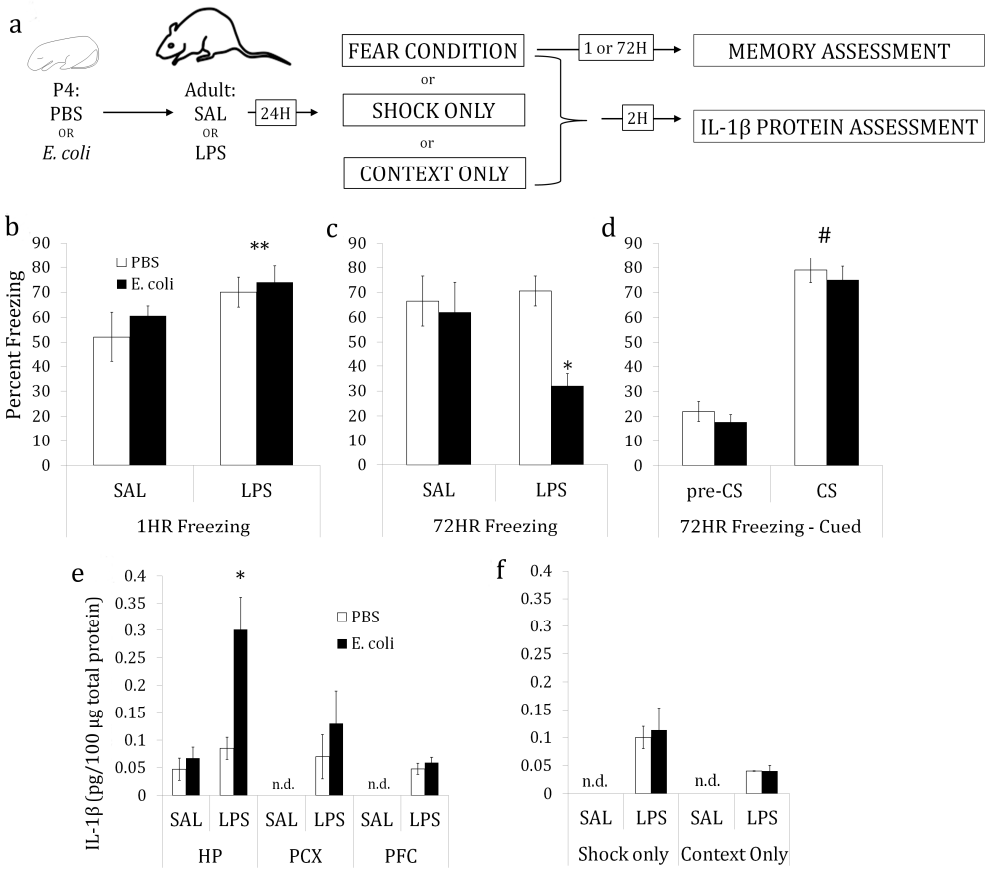

Figure 1: Learning increases IL-1β protein specifically within the hippocampus, and this increase is modulated by neonatal infection. a) Rats treated with PBS or *E. coli* on P4 were injected as adults with saline (SAL) or LPS, and fear conditioned 24 h later received Fear Conditioning, Shock only, or Context exposure only (n = 8/grp). Brains were collected 2 h later to assess IL-1β protein. b) Freezing to the context did not differ between groups at the 1 h test, although freezing was greater overall in LPS-injected rats (p<0.05). c) At the 72 h test, freezing to the context was significantly lowest in NI rats treated with LPS 24 h prior (*p<0.001). d) Freezing to the tone cue (CS) in an altered environment was significantly greater than the pre-CS period, but there was no neonatal group difference (#p<0.001). e) IL-1β was exaggerated within the HP of NI rats following LPS (*p<0.02). Moreover, IL-1β was detectable only in HP following SAL injection (**p<0.001), and concentrations were highest overall in HP (#p<0.001). *F*, Neither Shock nor Context alone produced the same exaggerated response within the HP of NI plus LPS rats, and concentrations were undetectable 24 h after SAL. Error bars indicated SEM.**

In a second group of rats from each group, brains were collected 2 h after fear conditioning to assess whether learning induces IL-1β protein within the HP, and whether this is altered by neonatal infection (n = 8/group). Figure 1e illustrates that IL-1β protein is markedly exaggerated within the HP of NI rats that received LPS 24 h before Fear Conditioning [main effects of neonatal treatment ($F_{(1, 31)} = 6.24$; $p < 0.02$) and adult injection ($F_{(1, 31)} = 6.21$; $p < 0.02$)], but not within either cortical region ($p > 0.05$). In a second experiment, adult rats from each neonatal group were injected with either SAL or LPS 24 h before an immediate 2 s Footshock only, or to a 5 min Context exposure only. Notably, LPS before either Shock alone or Context alone did not similarly exaggerate IL-1β within the HP ($p > 0.05$ for both) (Figure 1f). Importantly, a saline injection 24 h before either Shock or Context did not yield detectable IL-1β protein within the HP, indicating that learning is necessary to elicit the production of IL-1β

within the HP [ANOVA on ranks for all saline-injected rats (H = 24.1, df = 2, $p < 0.001$; Conditioned > Shock and Context)]. Finally, we assessed whether observed increases in IL-1β in the saline before conditioned group differed by brain region; there was a significant main effect of region ($F_{(1, 47)} = 29$; $p < 0.001$), with concentrations higher in HP than either PCX or PFC. Based on these data, we included only the HP of Fear Conditioned and Shock only groups for all further analyses.

2.3.2 Microglial but not astroglial activation marker expression within the hippocampus reflects the pattern of IL-1β

Microglia and resident macrophages are a major source of IL-1β within the brain. However, many other cell types, including astrocytes and neurons, can produce IL-1β within the brain (233). To determine whether microglia/macrophages are the source of IL-1β during non-pathological conditions, specifically during learning, we assessed expression levels of CD11b and GFAP, two activity-dependent surface antigens present on microglia/perivascular macrophages, and astrocytes, respectively, within the opposite hemisphere of hippocampi assessed for IL-1β in Figure 1. For CD11b, there were significant main effects of neonatal group (*$E. coli$ > PBS) and injection (**LPS >SAL) ($F_{(1, 31)} = 6.1$; $p < 0.02$, for each) after Fear Conditioning (Figure. 2a). In contrast, there were no significant effects of neonatal treatment on CD11b expression in rats that received LPS 24 h before Shock only ($p > 0.05$). However, there was a significant effect of LPS ($F_{(1, 31)} = 6.9$; $p < 0.01$). For GFAP, there were no significant group differences in either Fear Conditioned or Shock only groups ($p > 0.05$, for both; Figure 2b).

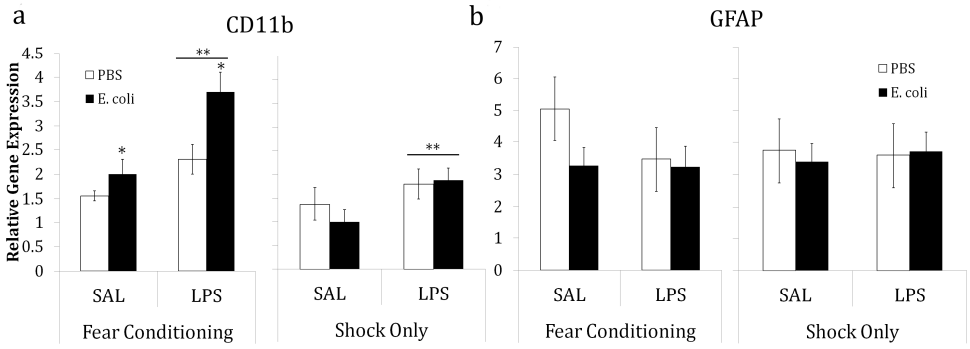

Figure 2: Microglial activation within the HP is exaggerated in NI rats, but only in response to learning. Rats treated with PBS or *E. coli* were injected as adults with SAL or LPS, and 24 h later received Fear Conditioning or Shock only (n = 8/group). Brains were collected 2 h later, and CD11b and GFAP were assessed. a) CD11b relative expression to GAPDH was increased by LPS ($p < 0.02$) and exaggerated within the HP of NI rats (overall effect of *E. coli*, *$p < 0.02$). Shock alone did not produce the same exaggerated response within the HP of NI rats, although there was a significant effect of LPS (**$p < 0.05$). b) GFAP expression did not significantly differ among groups, either following Fear Conditioning or Shock alone ($p > 0.05$ for both). Error bars indicate SEM.**

2.3.3 NI rats exhibit decreased neuronal inhibition within the hippocampus during learning compared with controls

Our preliminary data suggest that microglia are more reactive to LPS in NI rats. Microglial activity is modulated in part via direct contact with neurons and their surface-expressed and secreted products. For instance, CD200 interacts with CD200R on myeloid cells and CX3CL1 (fractalkine) can bind to its receptor (CX3CR1) on microglia (234, 235). We determined expression levels for each of these factors in the same HP tissues presented above in Figure 2. In the Fear Conditioned group, there was a significant effect of neonatal treatment on CD200 ($F_{(1, 31)} = 6.7$; $p = 0.01$), with expression lowest overall in NI rats, and significant effects of neonatal treatment ($F_{(1, 31)} = 15$; $p <$

0.001) and injection ($F_{(1, 31)} = 7.1$; $p < 0.02$) on CD200R (Figure 3a). In the Shock only group, there was a significant effect of injection on both CD200 and its receptor ($F_{(1, 31)} = 7.1$; $p < 0.02$, for both; SAL > LPS), but no neonatal group difference (Figure 3b). Thus, the decreased neuronal inhibition in NI rats compared with controls depends on learning. In contrast, there were no significant differences in CX3CL1 in either the Fear Conditioned (Figure 3c) or Shock only (Figure 3d) group; however, there was a significant effect of neonatal treatment on its receptor CX3CR1 ($F_{(1, 31)} > 5$, $p < 0.04$; PBS > *E. coli*, for both).

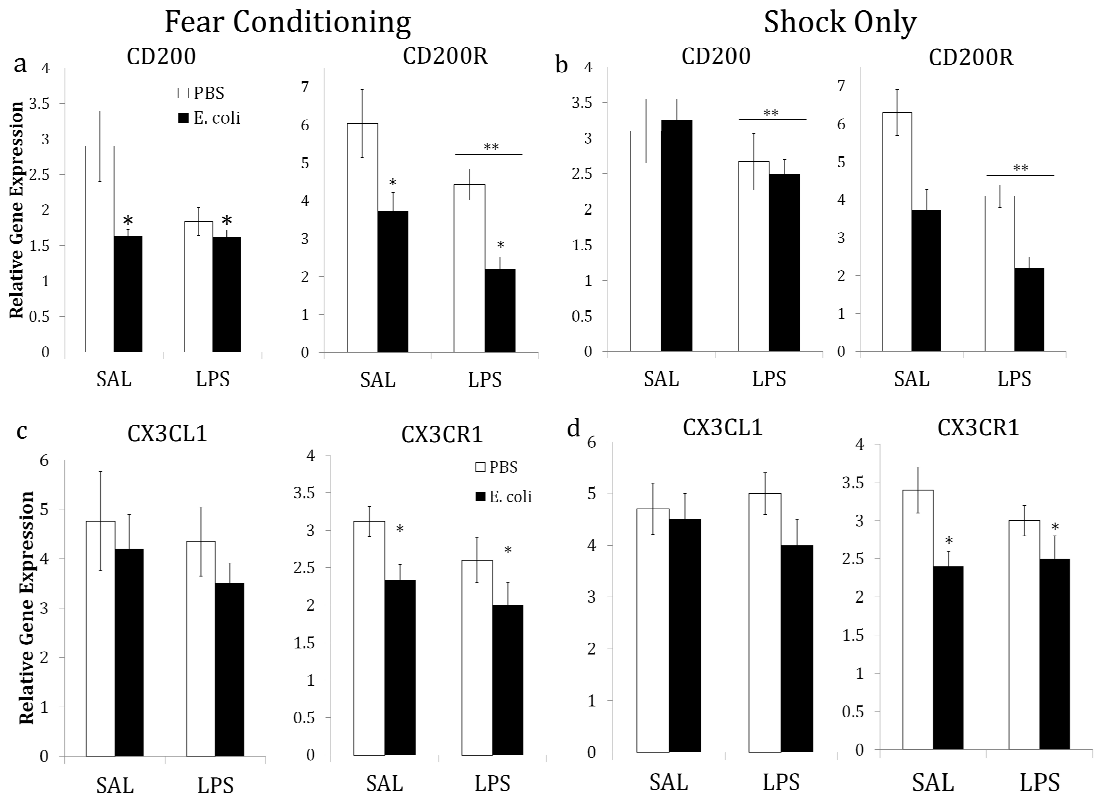

Figure 3: NI rats exhibit decreased neuronal inhibition within the HP compared with controls. Rats treated with PBS or *E. coli* on P4 were injected as adults with SAL or LPS, and 24 h later received Fear Conditioning or Shock only (*n*=8/group). Brains were collected 2 h later, and target genes were assessed. a) CD200

and its receptor (CD200R) were reduced overall in NI rats compared with controls in the fear conditioned group (*p<0.01 for both), whereas only CD200R was significantly decreased by LPS (p<0.03). b) There were no neonatal group differences CD200 or CD200R in the Shock only group; however, both genes were decreased by LPS (**p<0.02). c, d) Fractalkine receptor (CX3CR1) but not its ligand was reduced overall in NI rats in both Fear Conditioned and Shock only groups. (*p<0.05 for all, compared with PBS). Error bars indicate SEM.**

2.3.4 Hippocampal microglia from NI rats express greater levels of CD11b on a per-cell basis compared with controls

We have demonstrated that microglia show a marked increase in CD11b mRNA within the HP in response to the peripheral infection on P4, and that this increase is both sustained into adulthood, and exaggerated in response to LPS in NI compared with control rats (147, 227). Moreover, the number of microglia does not differ within the adult HP as a consequence of neonatal infection (165). Rather, the morphology of the cells is altered – microglial cell volumes are larger with shorter, thicker processes in adult rats infected as neonates compared with controls (165). These combined data suggest that changes in the function of microglia, rather than changes in number, may underlie the increased reactivity observed in adult NI rats, consistent with glial sensitization or priming.

To assess this directly, the HP was collected from adult rats in each neonatal condition. Before tissue harvest, rats were perfused with cold saline to eliminate infiltrating cells. Dissected hippocampi were brought to single-cell suspensions using Miltenyi's Neural Dissociation Kit (P) (Miltenyi Biotec) followed by myelin depletion and staining with APC-conjugated CD11b. Expression was quantified using a FACS

Canto II flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star) (Figure 4a). The mean fluorescence intensity (MFI) of CD11b expression on gated microglia (CD11b+) was significantly greater on a per-cell basis in NI (n = 10) compared with PBS (n = 9) rats ($t_{(17)}$ = 2.5; p = 0.02) (Figure 4b). Notably, the relative frequency of CD11b+ cells in our mixed cell population did not differ between groups. These data are pooled from five independent isolations of two animals per treatment group per day. In a separate experiment, myelin-depleted cells from rats in each neonatal treatment group were sorted into CD11b+ and CD11b- fractions using magnetic beads. A rightward shift can be seen in the population of CD11b+ cells from NI rats, indicating increased expression (vertical line is provided for comparison) (Figure 4c). Notably, the increased surface expression of CD11b in *E. coli* rats in both preparations indicates the increase observed in Figure 4b is not altered by the MACS enrichment step. Moreover, both increases were independent of any adult challenge, suggesting that surface antigen expression of microglia may be permanently altered as a consequence of the neonatal infection. Finally, a subset of sorted CD11b+ cells were stained with APC-conjugated CD11b/c and CD45 (a marker highly expressed on infiltrating macrophages), which revealed a large, dense cluster of $CD11b^{high}/CD45^{low}$ cells. In contrast, $CD11b^{high}$ cells that also stained brightly for CD45 accounted for only 4.2% of the population, indicating the perivascular macrophage population was quite small (Figure 4d).

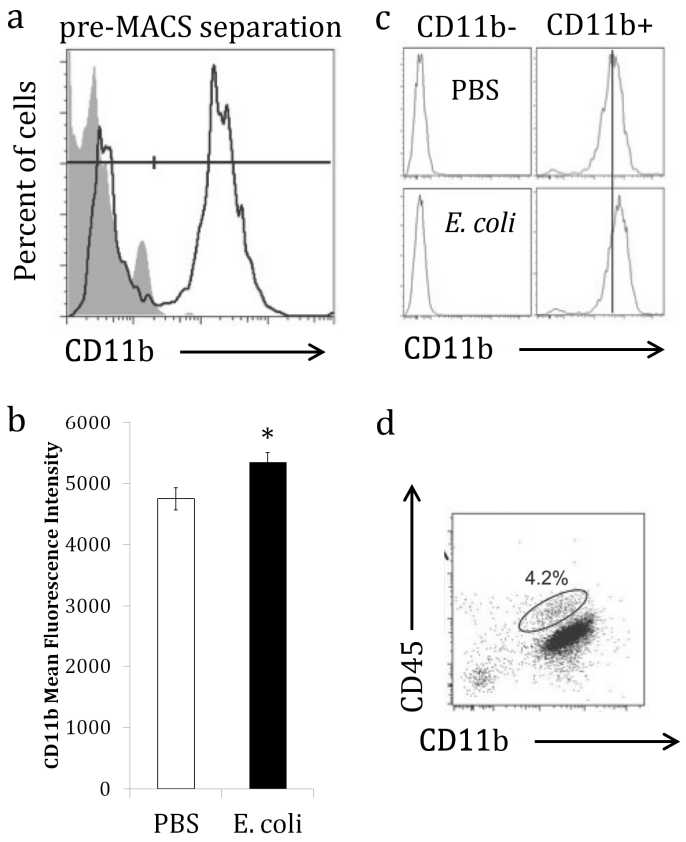

Figure 4: NI rats exhibit increased surface antigen expression of CD11b on isolated HP microglia. a) Adult rat HP were microdissected following cold saline perfusion and brought to single-cell suspensions using Miltenyi's Neural Dissociation Kit followed by myelin depletion. Myelin-depleted cells were stained with APC-conjugated CD11b and analyzed using flow cytometry. Representative presorted populations are shown as distinct peaks. An unstained control is overlaid for reference (shaded histogram). b) Myelin-depleted cells from rats in each neonatal treatment group were stained with APC-conjugated CD11b to assess expression on microglia independent of selection using magnetic beads, which could influence antigen expression. The bar graph shows greater average (±SEM) MFI for CD11b reactivity from NI (n=10) compared with PBS (n=9) rats (*p<0.05). c) Myelin-depleted cells from rats in each neonatal group were sorted into CD11b+ and CD11b- fractions using magnetic beads. Representative postsorted (MACS) populations are shown. Purity was consistently >93% CD11b+ and ~99% CD11b- for all samples. A rightward shift can be seen in the population of CD11b+ cells from NI rats, indicating increased expression (vertical line is provided for comparison). d) Sorted CD11b+ cells were stained with APC-conjugated CD11b/c and CD45 (a marker highly expressed on

infiltrating macrophages), which revealed a large, dense cluster of CD11b^{high}/CD45^{low}cells. In contrast, CD11b^{high} cells that also stained brightly for CD45 accounted for only 4.2% of the population, indicating the perivascular macrophage population was minimal.

2.3.5 Hippocampal microglia/macrophages from NI rats express greater IL-1β compared with controls

Next, we determined whether microglia from NI rats express more IL-1β to an LPS challenge *ex vivo* compared with controls. First, the HP was removed from untreated adult rats and a single-cell suspension was obtained using myelin depletion as described above. Isolated HP cells were magnetically sorted into CD11b+ and CD11b- fractions. HP CD11b+ and CD11b- fractions were then cultured with and without LPS (10 ng/ml) for 4 h. Immediately following culture, cells were lysed and total RNA was isolated for qPCR of relative IL-1β gene expression. IL-1β was only detected in the CD11b+ fraction, and its expression was increased following LPS in our group of unmanipulated control rats (Figure 5a). Next, we repeated this experiment using CD11b+ cells isolated from adult rats in each neonatal treatment group (n = 6 per group). There was a significant effect of neonatal treatment ($F_{(1,8)}$ = 8.2; p = 0.02); IL-1β expression was significantly higher in NI rats, both in response to media and to LPS (Figure 5b). This increase did not require an *in vivo* LPS challenge, suggesting that priming is intrinsic to microglia in NI rats and does not require an acute interaction with other cell types. While the basal increase in IL-1β in the NI plus media group may be a consequence of the isolation procedure itself, microglia are regardless more reactive in NI rats.

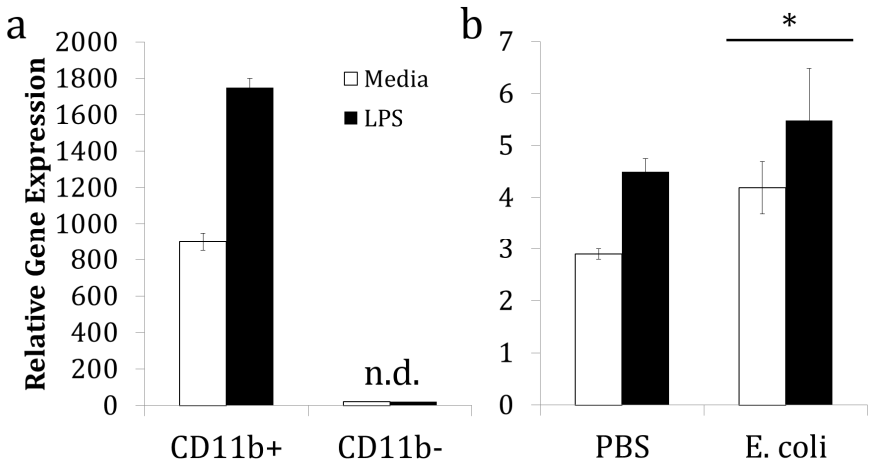

Figure 5: Isolated HP microglia from NI rats express exaggerated IL-1β mRNA *ex vivo*. a) Microglia were rapidly isolated from the adult HP of rats using myelin depletion and CD11b+ selection as described above in Figure 4. HP CD11b+ and CD11b- fractions were cultured with and without LPS (10 ng/ml) for 4 h at 37°C. Immediately following culture, cells were lysed and total RNA isolated for RT-PCR. Relative IL-1β gene expression to GAPDH is shown, which is increased in response to LPS and undetectable in the CD11b- population (*n*=2/group). b) Microglia were isolated from the adult HP of rats from each neonatal treatment. CD11b+ cells from NI rats express greater relative expression of IL-1β with or without LPS stimulation *ex vivo*, compared with controls (p*=0.02). The bars represent the average (±SEM) relative expression values for two rat HPs (pooled) per treatment group per day run as three independent experiments (*n*=6/group).**

2.3.6 The exaggerated increase in IL-1β *in vivo* in NI rats is microglial derived and requires learning

Our first experiment demonstrated that CD11b expression reflects the pattern of IL-1β protein within the HP (Figures 1, 2). Moreover, microglia from NI rats express exaggerated IL-1β *ex vivo* (Figure 5). However, exaggerated expression of both CD11b and IL-1β *in vivo* were only observed in conjunction with fear conditioning (Figures 1, 2). To determine whether microglia are the source of exaggerated microglia during

learning, we measured the expression of IL-1β from directly isolated CD11b+ cells following either Fear Conditioning or Shock alone. Adult rats from each neonatal treatment group were injected intraperitoneally with either saline or LPS 24 h before Fear Conditioning (n = 6-7/group) or Shock (n = 4-5/group), and brains were collected 1 h later following cold saline perfusion. The HP was extracted and microglia were isolated using myelin depletion and CD11b+ selection as described above. Cells were immediately lysed and total RNA was isolated for qPCR of relative IL-1β gene expression. Following fear conditioning, there were significant effects of group ($F_{(1,26)}$ = 4.7; p = 0.041) and injection ($F_{(1,26)}$ = 6.1; p = 0.021) (Figure 6a), with expression highest in the NI plus LPS group. In contrast, there were no significant effects of neonatal treatment in the Shock only group (Figure 6b). In a separate control experiment, CD11b- cells were selected from the HP of rats in each neonatal treatment that received Fear Conditioning 1 h before killing as above (n = 2/group), to confirm that microglia/macrophages are the primary source of IL-1β in response to fear conditioning. Figure 6c illustrates that IL-1β mRNA was completely undetectable in the population of CD11b- cells, consistent with our previous *in vitro* findings (Figure 5b). Finally, to further characterize the CD11b- population, we assessed GFAP and CX3CL1 mRNA; both were highly expressed in CD11b- cells but not CD11b+ cells (Figure 6d), indicating the CD11b- population contained astrocytes and neurons, respectively, which were viable and responsive to LPS.

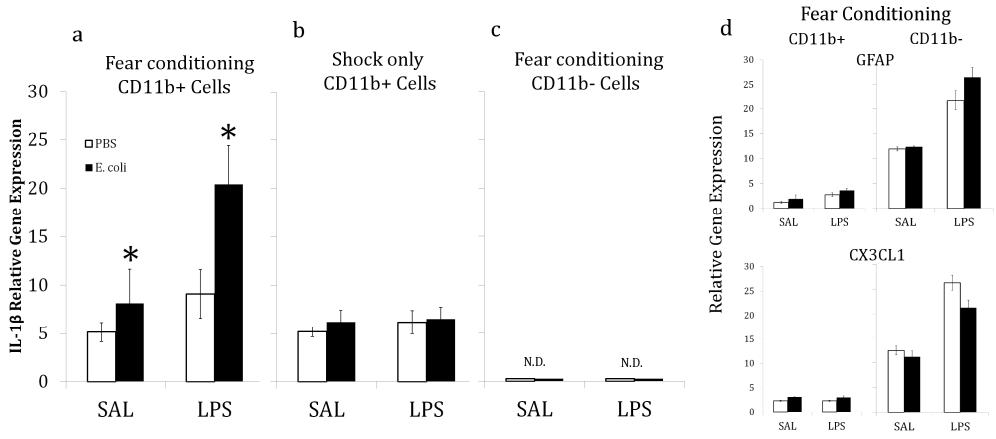

Figure 6: HP microglia isolated 1 h after behavioral experience express exaggerated IL-1β mRNA in NI rats compared with controls, but only following learning. Rats treated with PBS or *E. coli* on P4 were injected as adults with SAL or LPS, and received Fear Conditioning or Shock only 24 h later. Microglia were rapidly isolated 1 h later using myelin depletion and CD11b$^+$ selection as described above in Figure 4. Isolated microglia were immediately lysed and total RNA isolated for RT-PCR. Relative IL-1β gene expression to GAPDH is shown. a) IL-1β mRNA expression was higher overall in NI rats (*p=0.04) and higher in response to LPS in both groups (p=0.02) (n=6-7/group). b) There were no group differences 1 h following Shock alone (p>0.05; n=4-5/group). c) IL-1β mRNA was undetectable in CD11b$^-$ cells collected from a separate group of rats in each condition that also received fear conditioning (n=2/group). d) GFAP and fractalkine/CX3CL1 were robustly expressed in CD11b$^-$ cells, indicating this population contained astrocytes and neurons, and was viable and responsive to LPS. Error bars indicate SEM.

2.3.7 Minocycline prevents the exaggerated hippocampal IL-1β response, and the memory impairment in adult rats infected as neonates

Our final goal was to prevent the memory impairment in NI rats by pharmacologically inhibiting microglia. To do this, we used the microglial inhibitor MINO, which we administered via oral gavage to mimic the clinical route of administration and to avoid the visceral irritation that occurs following intraperitoneal injection (our unpublished observations). In our first experiment, rats from each

neonatal treatment group were injected with LPS as adults, and fear conditioned 24 h later (Figure 7a). Rats also received a loading dose of MINO (50 mg/kg) or its vehicle (water) in a volume of 5 ml/kg via oral gavage 12 h before LPS injection, and received a second dose (water or 25 mg/kg) 1 h before injection. This dosing regimen significantly decreases proinflammatory cytokine production by microglia (236). Contextual memory was assessed 72 h after conditioning in one-half of the animals in each group (n = 10/group MINO treated, and n = 8/group water treated). In the remaining animals (n = 8-10/group), brains were collected 2 h after fear conditioning to assess IL-1β protein within the HP. For freezing behavior, there was a significant group by drug interaction ($F_{(1,35)}$ = 7.3; p = 0.01). Freezing was lower in NI plus water rats compared with all other groups (p < 0.03 for all), and MINO given before LPS completely reversed this (Figure 7b). For IL-1β protein, there was a significant interaction as well ($F_{(1,35)}$ = 4.9; p = 0.034); NI rats had higher IL-1β concentrations than control when treated with water before LPS (p = 0.01), whereas MINO given before LPS prevented this increase (Figure 7d, left panel).

In a second group of rats, we determined whether MINO given just before fear conditioning but after LPS would similarly restore memory in NI rats (Figure 7). With the paradigm, a normal inflammatory response to LPS was allowed to occur before MINO administration, allowing us to confirm our hypothesis that exaggerated microglial-derived IL-1β production at the time of learning is critical for the observed memory impairment. Thus, rats from each neonatal treatment were injected with LPS as

adults, and fear conditioned 24 h later as before. All rats also received MINO 12 h and again 1 h before fear conditioning (12 and 23 h after LPS injections). There were no significant differences among groups in percentage freezing to the context (Figure 7c), indicating that MINO before conditioning also prevented the memory impairment in NI rats, even in the face of prior LPS. For IL-1β protein, there was also no group difference (Figure 7d, right panel), indicating that MINO before fear conditioning prevented the exaggerated increase we had previously observed in NI plus water rats (left panel).

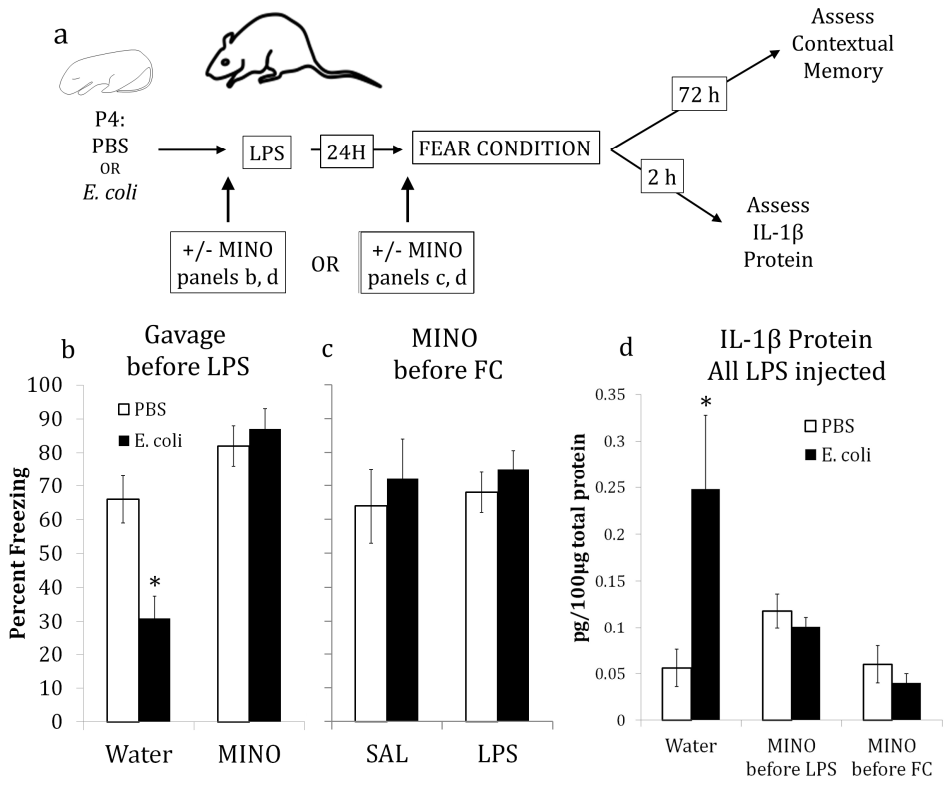

Figure 7: Inhibiting microglia before LPS or before learning prevents the memory impairment and exaggerated IL-1β response in NI rats. a) Adult rats treated on P4 with PBS or *E. coli* were injected with LPS 24 h before fear conditioning. A subset of animals were killed 2 h later to assess HP IL-1β protein, and the remaining

animals were tested for memory at 72 h. b) Rats in each neonatal treatment group (n=10/group) received water or MINO 12 h and again 1 h before LPS in experimental paradigm illustrated in *a*. Freezing was significantly lowest in NI rats that received water before LPS (*p <0.03), but this decrease was prevented by MINO. c) A separate group of rats in each neonatal treatment group (n=10/group) received MINO 12 h and again 1 h before Fear Conditioning (FC), but after LPS the previous day. A SAL-injected group was also included to ensure that MINO alone did not impair learning. There were no differences between groups. d) IL-1β protein was significantly highest in NI rats that received water before LPS (*p<0.03) (left-most bars), but this increase was prevented by MINO, administered either before LPS (center bars), or in a separate group, after LPS but before FC (right-most bars). Error bars indicate SEM.

Because MINO can have sedative properties, we included a group of saline-injected rats in the latter experiment to confirm that MINO itself does not impair learning; freezing levels were comparable with LPS-treated and water-gavaged rats in the previous experiment. Finally, we also assessed activity levels in a separate group of untreated adult rats (n = 5/group, water vs. MINO) using an open field. This test was performed 1 h after the second gavage, at the time that fear conditioning would normally occur. Consistent with the results for fear conditioning, MINO did not alter overall activity in the open field compared with water-treated controls (total activity counts, mean ± SEM: water, 1000 ± 230; MINO, 1090 ± 220; $p > 0.05$; data not shown).

2.4 Discussion

Bacterial infection early in life leads to marked HP-dependent long-term memory impairments in adulthood, but only when combined with a "second hit," an LPS challenge before learning, consistent with our previous findings (149). The primary goal of this study was to determine the cellular source of HP IL-1β in NI rats, which we have

demonstrated is exaggerated in response to LPS and causally linked to the memory impairment (147). We sought to determine the mechanism by which long-term changes in immune reactivity within the brain can endure in response to an initial insult, potentially throughout life, and thereby lend insight into the environmental risk factors for neurodegenerative and inflammatory disorders that exhibit a broad spectrum of prevalence and severity that cannot be explained by genetics alone.

We show for the first time that CD11b+ cells are the sole source of IL-1β in response to normal HP-dependent learning and that early-life events can significantly modulate learning-dependent cytokine activity within the HP via a specific, enduring effect on microglial function. As noted previously, our population of CD11b+ cells does not exclude resident perivascular macrophages; however, this population is quite small (~4%). The resident microglia are functionally primed within the HP as a consequence of the early-life infection; specifically, microglia from NI rats express greater CD11b on a per-cell basis and produce more IL-1β *ex vivo* compared with microglia from controls. Furthermore, blocking microglial activation with MINO prevents both the sensitized IL-1β response and the cognitive impairment in NI rats. Importantly, the sensitized IL-1β response 24 h after LPS in NI rats *in vivo* requires a learning experience, as Footshock or Context exposure alone does not elicit the same exaggerated response. Moreover, IL-1β protein was completely undetectable within cortical regions of saline-injected rats that experienced fear conditioning, whereas concentrations were low but detectable in the

HP of all rats. Thus, the learning experience itself induced IL-1β expression in a HP-restricted manner. Finally, preventing microglial activation during learning prevented the memory deficit in NI rats, even following an adult LPS challenge.

There is a significant literature documenting the role of IL-1β in both normal memory and in memory disruption (52). Physiological levels of IL-1β are required for memory, whereas aberrant levels (either too low or two high) become detrimental and are strongly associated with disorders like AD. IL-1β mRNA increases as a consequence of learning (57, 237). Furthermore, IL-1 type 1 receptor expression in astrocytes is important for HP-dependent LTP and long-term memory (60). However, the source of IL-1β during learning has not been demonstrated, and neurons, astrocytes, and microglia, among other cells, are thought to produce IL-1β within the brain (233). We show that learning, but not Shock or Context exposure alone, induces IL-1β protein specifically within the HP, and that its expression is markedly exaggerated in NI rats given LPS 24 h before. Moreover, we replicated this pattern for mRNA in purified CD11b+ cells (Figure 6), whereas IL-1β mRNA was completely undetectable in the population of CD11b- cells, regardless of behavioral experience (Figures 5, 6). Together, we have demonstrated that microglia are primed as a consequence of neonatal infection and that this priming leads to aberrant production of IL-1β specifically during learning in adulthood. Importantly, these data suggest that the resident microglia of the HP are a necessary component of normal memory formation.

The vulnerability factors leading to cytokine dysregulation within a given individual are not well understood, as the majority of research on IL-1β and cognition has been conducted in genetic mouse models either entirely lacking IL-1 or its receptor, or with overexpression of its antagonist, IL-1ra (52). Similarly, glial function has often been explored in models of induced injury (e.g., stroke, trauma) in which the precipitating event is intrinsic to the model, creating questions about etiology that are difficult to answer. There is compelling evidence from the neurodegeneration and aging literatures that microglia become sensitized or primed following initial activation (by largely unknown factors), which contribute to neuronal dysfunction or death (238-241). Notably, primed glia do not constitutively overproduce proinflammatory mediators. However, the response by primed glia to a subsequent challenge (e.g., systemic infection) is significantly exaggerated, compared with resting/quiescent glia that receive the same challenge (239). This pattern of sensitization is similar to the phenotype we observe in our "two-hit" model. However, our data provide the first evidence to our knowledge that early-life experience can change microglial function within the brain well beyond the initial insult, perhaps for the remainder of the life span. Indeed, we have demonstrated that NI rats exhibit accelerated cognitive decline in middle age (16 months), independent of acute immune challenge, a change that is linked to exaggerated aging-related glial sensitization (221). Thus, aging itself, and its impact on glial reactivity, acts as a "second hit" only in rats made vulnerable by neonatal infection.

Increasing evidence suggests a role for microglia in normal synaptic plasticity mechanisms within the adult brain, including dendritic spine remodeling and elimination (106, 242-244). These cells are active and dynamic even when "quiescent" (103) (e.g., they continually survey their microenvironments by extending and contracting processes into nearby synapses with a frequency that is activity dependent (107, 245)). They have receptors for multiple neurotransmitters, including those important for learning and memory (e.g., ATP, norepinephrine, glutamate) (246). The requirement of learning to observe the exaggerated IL-1β response in NI rats 24 h after LPS *in vivo* implies a neuronal-glial interaction. CD200 and CX3CL1 are both expressed by neurons within the brain and tonically inhibit microglia via CD200R and CX3CR1, respectively (234, 235). Notably, these inhibitory factors decrease during aging and in neurodegenerative disorders like AD, likely contributing to increased microglial reactivity (247-249). Mice deficient in fractalkine receptor, CX3CR1, exhibit enhanced Tau pathology within the HP that is dependent upon microglial-derived IL-1β (250). In our study, CX3CL1 did not differ by group, whereas its receptor was decreased basally in NI rats, implicating a change at the level of microglia. In contrast, CD200 and its receptor were both reduced basally in NI rats, but only following fear conditioning. These data suggest there may be a rapid chemokine role for CD200 in synaptic plasticity similar to that already described for CX3CL1, which plays a role in AMPA receptor function in active synapses (81), but this hypothesis remains to be explored.

The mechanism by which learning leads to microglial production of IL-1β within the HP also remains to be determined. One notable finding in this study was that MINO before FC did not impair memory, given that constitutive levels of IL-1β are considered vital for memory formation as discussed previously. However, MINO did not completely suppress IL-1β in either group of rats (Figure 7d). Thus, while the protein concentrations are low, they are detectable, in contrast to saline-injected rats that do not receive fear conditioning (Figure 1, Panels e and f). Therefore MINO-treated rats likely maintain a threshold amount of IL-1β needed for memory consolidation. If true, this suggests that MINO prevents the production of IL-1β in response to an inflammatory stimulus (LPS), but not in response to a learning-induced stimulus/signal, which may be neurotransmitter/hormone mediated. Finally, there is evidence that MINO increases rather than suppresses the release of IL-1α (251, 252), which has been implicated in LTP. Importantly, these potential mechanisms of memory formation in the face of MINO are not mutually exclusive and remain to be further explored.

In closing, we demonstrate for the first time that CD11b+ cells are the primary source of IL-1β within the HP during normal learning. Minocycline is an imperfect microglial inhibitor with nonspecific anti-inflammatory properties. However, our pharmacological and behavioral experiments combined with the finding that IL-1β was produced exclusively in CD11b+ cells directly as a consequence of learning strongly point to a critical role of microglia in normal learning. Most importantly, we have

identified an early-life risk factor that alters the normal, activity-dependent "set point" of IL-1β via a specific, enduring impact on microglia. Microglia derive from primitive yolk sac macrophage precursors, which are of mesodermal origin and enter the neuroectoderm during embryogenesis, a founding colony of which proliferates to populate the entire developing parenchyma (160). Thus, early-life events may exert enduring impacts on the brain and behavior of organisms via an impact on the function of these resident, long-lived immune cells. The hypothesis that the early-life environment is especially critical in determining later risk for disorders like AD is supported clinically (253, 254). Furthermore, in young adult humans, there is significant variability in learning and memory impairments following low-dose endotoxin treatment that is inversely correlated to induced circulating cytokine levels (255). Our data provide valuable insight into the mechanisms underlying such variability and suggest the interesting possibility that changes in cognition following low-dose endotoxin challenge in young adults may serve as a novel biomarker for increased risk of later-life dementia. This possibility, as well as the role of microglia and their secreted molecules in normal synaptic plasticity mechanisms more generally, warrants further research.

3. Neonatal infection modulates behavioral flexibility and hippocampal activation on a Morris Water Maze task

3.1 Introduction

Spatial navigation is sensitive to disruptions in cytokine signaling within the hippocampus, as described above, in Morris Water Maze studies with transgenic and knockout mice (55-57, 59, 60). As the previous experiments (Chapter 2) demonstrated, neonatally infected rats have altered IL-1β signaling within the hippocampus as well as primed microglia that remain reactive into adulthood. In addition to memory impairments in fear conditioning in adulthood following neonatal infection (108, 147-149), we have demonstrated that neonatally-infected male rats acquire a platform location more quickly than controls on a Morris Water Maze task in young adulthood. Aged male rats that were treated with *E. coli* on P4, however, have impaired memory for the platform location 24HR after testing (221). We have also demonstrated alterations at the cellular level in neonatally-infected rats. *E. coli* infection on P4 significantly reduced proliferation of neurons in the CA1 and CA3 sub-regions of P6 pups and reduced the maturation and integration of neurons in the CA1, CA3 and DG regions of P33 rats (165). Nevertheless, our previous work has not examined the effects of *E. coli* infection on hippocampal neuronal networks. The persistent changes in neurogenesis following early-life infection may be indirect evidence that hippocampal circuitry is enduringly altered in these rats.

Neuronal activation during behavioral tasks can be measured in a variety of ways. Immediate early gene (IEG; e.g. *Arc*/*Arg 3.1*, *Zif268*, *cFos*) expression is a non-invasive technique for measuring cellular activation in the brain. Protein expression of IEGs has a well-defined time course; thus, assessing protein expression at specific times following behavior reveals the populations of neurons that were activated during a given behavioral task (9, 256-260). The intracellular kinetics of activity-regulated cytoskeleton-associated (*Arc*) mRNA and protein are well-characterized (9, 261, 262) and extensive work on Arc at both the mRNA and protein levels demonstrates a significant role for its activity-dependent transcription and translation as a mechanism for synapse-specific plasticity (for reviews, see 263, and 264). Inhibiting Arc protein expression impairs long-term potentiation (LTP) and memory consolidation (265). Thus, we characterized Arc protein in this study as both a time-sensitive readout of neuronal activation and a representation of possible plasticity during a learning and memory task.

In light of the growing literature on enduring cognitive changes following perinatal infection or inflammation, this study examines the effects of bacterial infection on spatial learning and, indirectly, its underlying neural correlates. We assessed the impact of neonatal *E. coli* infection on water maze acquisition and memory in adulthood, and measured Arc expression following the memory probe to examine hippocampal activation patterns in rats exposed to bacterial infection early in life, along with age-matched controls. Based on the acquisition and memory behavior that we observed and

the potential for neonatal infection to alter many brain regions and not solely the hippocampus, we then examined reversal learning acquisition and memory on the water maze task to assess cognitive flexibility in our neonatally-infected rats.

3.2 Materials and Methods

Specific animal, apparatus and procedural details appear below in the General Methods section.

3.2.1 Experiment 1

The goal of this experiment was to test the effects of neonatal infection on a challenging paradigm of minimal training on the Morris Water maze (MWM) task in adulthood. We trained the rats with limited, minimal exposure to the apparatus and, thus, increased the difficulty of the task compared to training over a greater number of days or with more trials per day. We trained neonatally-infected and control rats for 3 days, 6 trials per day, to assess memory for a platform location after training. On the third day, half of the trained animals (n=36) were tested on a memory probe trial 2HR following their final training trial. The other half of the group (n=36) was tested on the probe trial 48HR following their last training trial. One hour after their respective probe trials, rats were taken for euthanasia. In addition to the trained rats, we assessed the effect of a single experience in the MWM on Arc expression in the DG, compared to expression after repeated experiences during training and testing. These rats were given

a single 60s trial in the pool without a submerged platform. One hour after their single trial, rats were taken for euthanasia.

3.2.2 Experiment 2

Based on our findings in Experiment 1, we assessed memory performance following extended training on the MWM task, increasing both trials per day and number of days of training. Rats received 5 days of 10 trials per day during training. One hour after a 48HR probe trial (60s), rats were euthanized and brains were collected. We assessed a separate group of rats that was "yoked" by latency to the trained rats to examine the importance of learning on neuronal activation in the DG during a hippocampal-dependent task. Yoked rats were treatment- and latency-matched to rats from the Extensive Training group. All yoked trials were conducted without a submerged platform, allowing for a similar experience in the environment without the act of learning. One hour after a memory probe trial (60s trial 48HR after last training trial), rats were euthanized and brains were collected.

3.2.3 Experiment 3

We next assessed cognitive flexibility with a reversal task paradigm on the MWM. Once rats were trained to the first platform location (5 days X 10 trials per day, as in Experiment 2) and tested on memory probe trials, the platform was moved to another location in the opposite quadrant (SE) of the pool. Rats were trained for 3 days of 10 trials per day on the new platform location until they reached criterion (less than 10s

average escape latency). They were tested 48HR after the last trial for memory for the reversal platform location. Rats were taken 1 hour after the 48HR probe trial for brain collection. The reversal task was assessed in 2 separate groups of rats (total n =16) and their data for acquisition, reversal and the 48HR probe trial for the reversal platform location were pooled.

3.2.4 General Methods

3.2.4.1 Animals

Adult male and female Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN) and were pair housed for breeding after a week of acclimation to the facility. Female breeders were visually examined daily for confirmation of pregnancy, and male breeders were removed from cages prior to the birth of pups (P0). All rats were housed in individually ventilated polypropylene cages with *ad libitum* access to food and filtered water. The colony was maintained at 22°C on a 12:12h light:dark cycle (lights on at 0700 h). Sentinel animals were housed in the colony room and screened periodically for the presence of common rodent diseases; all screens were negative. All experiments were conducted with protocols approved by the Duke University Institutional Animal Care and Use Committee.

3.2.4.2 Neonatal Manipulations and Bacterial Cultures

All litters were culled on P4 to a maximum of 10 pups/litter, retaining 2 female and as many male pups as possible. The females were retained to prevent single sex

litters, but all female pups were euthanized at weaning (P21). All litters were born within 1 week of each other, and all studies were limited to males, limiting the conclusions about the data to males alone. *Escherichia coli* culture (ATCC 15746; American Type Culture Collection, Manassas, VA) vial contents were hydrated and grown overnight in 30ml of brain-heart infusion (BHI; Difco Labs, Detroit, MI) at 37°C. Cultures were aliquoted into 1ml stock vials supplemented with 10% glycerol and frozen at -20°C. One day before injections, a stock culture was thawed and incubated overnight in 40ml of BHI at 37°C. The number of bacteria in cultures was read using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT) and quantified by extrapolating from previously determined growth curves. Cultures were centrifuged for 15 min at 4000rpm, the supernatants were discarded, and the bacteria were re-suspended in the dose-appropriate volume of sterile Dulbecco's PBS (Invitrogen Corp., Carlsbad, CA). Male pups were injected subcutaneously using a 30G needle on P4 with either 0.1×10^6 colony forming units (CFU) of live bacterial *E. coli*/g suspended in 0.1 ml PBS or 0.1 ml PBS. All pups were removed from the mother at the same time and placed into a clean cage with bedding, injected individually, and returned to the mother as a group. Elapsed time away from the mother was less than 5 min. All pups from a single litter received the same treatment due to concerns of possible cross-contamination from *E. coli*. All injections were given between 1300 and 1430h. To control for possible litter effects, a maximum of 2 pups per litter were assigned to a single experimental group.

Pups were weaned on P21 into sibling pairs and remained undisturbed until adulthood. In adulthood, all male rats were trained and tested between ages P60 and P90 on the behavioral task.

3.2.4.3 Behavior

3.2.4.3.1 Apparatus

The water maze task consisted of a black circular pool approximately 1.8m in diameter and filled with room temperature water. A circular platform 15.5cm in diameter was submerged 2cm below the surface of the water in the center of one of the pool quadrants, and the water was clouded by black non-toxic water-based tempera paint to obscure the platform location. The pool was located in a well-lit room (approximately 5.8m x 2.6m in dimension) with salient extra-maze cues, such as a table with a computer, large black stripes adhered to two walls, shelving that contained large objects and the experimenter who sat in a chair near the computer.

3.2.4.3.2 Water Maze Training and Testing

For three days prior to behavioral testing, all rats were weighed and handled for approximately 60 seconds each per day. Testing was conducted over several days, depending on the training paradigm. On the first day, rats were habituated to the pool and the platform. On the following days, rats were trained for 6 to 10 trials per day to find the platform in the pool in a constant location (center of NW quadrant). On each training trial, the rat started at a random start location (N, S, E, W, SE, SW, NE) and was

allowed to swim until climbing onto the escape platform or until 60 s had elapsed, at which point it was guided to the platform. After 15 s on the platform, the rat was dried with a towel and placed in a holding cage for a 5-min inter-trial interval. After the final training day, rats were given a memory probe test either 2HR or 48HR after the final trial, depending on the paradigm. For the probe trial, the platform was removed from the pool and the rats swam for 60s. One hour after their respective probe trials, rats were taken for euthanasia.

Memory probe tests are reported as difference scores, calculated by assessing the time spent in the Target quadrant (previously containing the platform) and subtracting the time spent in the quadrant with the next highest value. If the rat spent more time in the Target quadrant than any other quadrant, the difference score is positive. If the rat spent more time in a quadrant other than the Target, the difference score is negative. Each group's score is an average of all the difference scores from the rats in the treatment group. The difference score is a conservative measure of performance in the MWM test, as it requires animals to be both accurate and precise in their identification of the target quadrant.

3.2.4.4 Tissue Harvest

Rats were deeply anesthetized with ketamine-xylazine cocktail and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M PBS. Brains were post-fixed for 24h in 4% paraformaldehyde in 0.1M PBS and cryoprotected

in 30% sucrose for at least 3 days and then quickly frozen in -30°C isopentane. 40-µm coronal sections were cut through the dorsal and ventral hippocampus in a -20°C cryostat using the atlas of Paxinos and Watson (266) as a guide. Sections were stored at 4°C in cryoprotectant until immunohistochemistry was performed.

3.2.4.5 Immunohistochemistry

3.2.4.5.1 Fluorescence Staining

In Experiment 1, we examined Arc protein expression in one series of brain slices and NeuN (neuronal nuclei) protein in another series double-labeled with a cell proliferation marker not assessed here. Free-floating sections were first rinsed for 3x5 min in 0.01M phosphate buffered saline (PBS) and also rinsed before each subsequent step, except between the blocking and primary antibody steps. Next, sections were washed in 50% methanol for 30 min. Sections were then blocked for another 30 min in 5% normal goat serum and 0.3% Triton-X to block and permeabilize, respectively, in PBS (blocking buffer). Sections were then incubated overnight at room temperature in Arc primary antibody (1:1000, rabbit polyclonal, Wako Pure Chemical Industries, Ltd., Osaka, Japan) or NeuN primary antibody (1:500, mouse monoclonal, Chemicon International, Temecula, CA USA) in blocking buffer. The following day, sections were incubated for 2 h at room temperature in a solution of goat anti-rabbit antibody bound to AlexaFluor 568 (Arc) or goat anti-mouse antibody bound to AlexaFluor 488 (NeuN) (both 1:200, Invitrogen, Grand Island, NY, USA) in blocking buffer. After PBS washes,

sections were mounted on gel-coated slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA). Coverslips were adhered to the slides using nail polish.

3.2.4.5.2 Diaminobenzidine (DAB) Staining

In Experiments 2 and 3, we did not perform double-label staining. Thus, we performed immunohistochemistry with a chromogen appropriate for a light microscope (DAB) for all subsequent experiments. Free-floating sections were first rinsed for 3x5 min in 0.01M phosphate buffered saline (PBS) and also rinsed before each subsequent step, except between the blocking and primary antibody steps. Next, sections were washed in 50% methanol for 30 min. Sections were then quenched in 0.6% hydrogen peroxide for 30 min and then blocked for another 30 min in 5% normal goat serum and 0.3% Triton-X to block and permeabilize, respectively, in PBS (blocking buffer). Sections were then incubated overnight at room temperature in Arc primary antibody (1:1000, rabbit polyclonal, Wako Pure Chemical Industries, Ltd., Osaka, Japan) in blocking buffer. The following day, sections were incubated for 2 h at room temperature in a solution of biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA USA) in blocking buffer. The Avidin-Biotin Complex (ABC) method was used to bind a complex of streptavidin-biotin peroxidase to the secondary antibody (1 h incubation), which was then developed with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) for 15-45 min to produce a colorimetric stain.

Sections were mounted on gel-coated slides, dehydrated and coverslipped with Permount.

3.2.4.6 Cell Quantification

Fluorescent images were visualized using a Zeiss 510 Metaseries inverted laser scanning confocal microscope (representative images shown in Figure 8a and 8b). Slices were continuously scanned from top to bottom (approximately 20μm) exhaustively at 40X through the DG and at two sites (250μm x 250μm) in the CA1 to count Arc-positive cells. Five sections were counted for each rat and each section was at least 200 micrometers from the previously counted section. The counts from each individual section were combined to create a single Arc-positive cell count value per rat.

DAB-developed images were visualized with a light microscope (representative images shown in Figure 8c and 8d). Quantification of Arc-positive cells in the granule cell layer of the dentate gyrus in the hippocampus was performed using a Nikon Eclipse 80i microscope on a Dell PC running StereoInvestigator software (MBF Bioscience, Inc., Williston, VT, USA). The boundaries of the dentate gyrus were traced using this software at a magnification of 10X and Arc-positive cells in the region of interest were counted at 40X. Five sections were counted for each rat and each section was at least 200 micrometers from the previously counted section. The counts from each individual section were combined to create a single Arc-positive cell count value per rat.

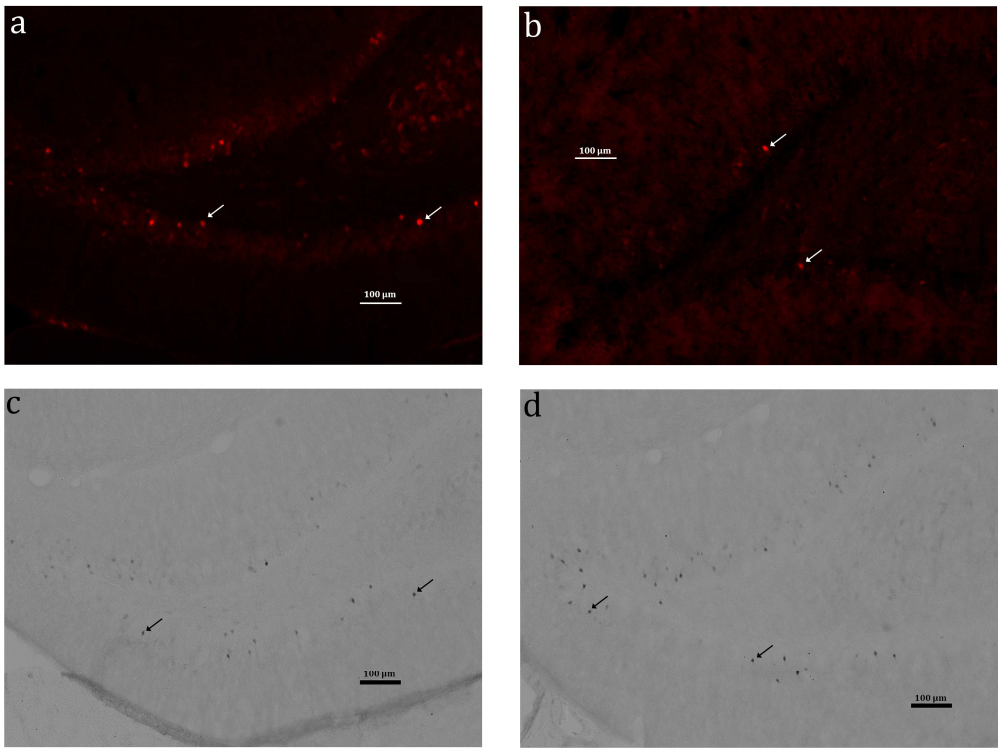

Figure 8: Representative images from immunohistochemical staining. Each image shows the dentate gyrus in one hemisphere at 10X magnification. The scale bars indicate 100µm and the arrows indicate representative Arc+ cells. a) DG from a minimally trained control rat after the 48HR probe. b) DG from a minimally trained neonatally-infected rat after the 48HR probe. c) DG from an extensively trained control rats after the 48HR probe. d) DG from an extensively trained neonatally-infected rat after the 48HR probe.

3.2.4.7 Statistical Analyses

Behavioral training was analyzed with a repeated-measures two-way ANOVA to analyze latencies in the water maze between treatment groups over several days of training. Memory probe tests and cell counts were analyzed with *t*-tests, comparing PBS and *E. coli* groups. *F* and *t* values for each analysis are reported in the Results section.

3.3 Results

3.3.1 Experiment 1

3.3.1.1 Neonatal treatment did not affect platform acquisition on the MWM during minimal training. However, neonatally-infected rats performed better than controls during a 48HR memory test.

Minimal training on the MWM tested the effects of neonatal infection on a challenging spatial navigation task. Both neonatally-infected and control rats acquired the platform location during minimal MWM training at the same rate and improved over days of training ($F_{(1,76)}$=91.3, p<0.001) (Figure 9a). Both groups performed equally well at the 2HR memory probe test ($t_{(38)}$= −1.28, p=0.207) (data not shown), but neonatally-infected rats had significantly better memory at the 48HR probe ($t_{(30)}$= −4.83, p=0.026) (Figure 9b).

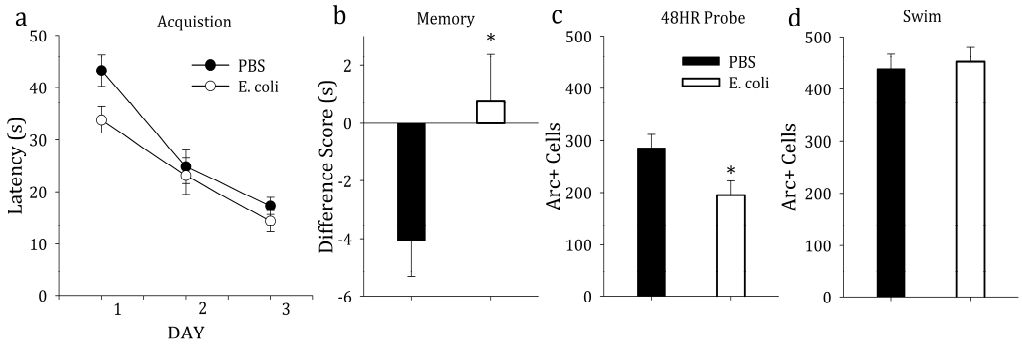

Figure 9: Neonatally-infected rats have better memory and decreased neuronal activation in the DG following minimal training. a) Both groups of adult rats (PBS- vs. *E. coli*-treated on P4) acquired the platform location on the water maze task equally well with minimal training ($F_{(1,76)}$= 9.13, p<0.001). b) Neonatally-infected rats had significantly better performance on the memory probe at 48HR ($t_{(30)}$= -4.83, p=0.026). c) Arc protein in the DG of neonatally-infected rats was significantly lower

at the 48HR probe than in PBS-treated control rats ($t_{(29)}$=2.47, p=0.020). There were no differences in Arc protein expression in the CA1 (not shown). d) After a single trial (SWIM), both treatment groups had equal Arc expression in the DG ($t_{(25)}$ = -0.32, p=0.75) and greater average expression compared to trained rats.

3.3.1.2 Neonatally-infected rats had significantly less Arc activation in the DG during the 48HR probe following minimal training.

During the 48HR memory probe test after minimal training, neonatally-infected rats had significantly fewer Arc-positive cells in the DG compared to controls ($t_{(29)}$=2.47, p=0.020) (Figure 9c). During the 2HR memory probe test after minimal training, there were no significant differences in Arc expression between groups ($t_{(37)}$=1.79, p=0.082; data not shown). In the CA1 region, both groups had equal Arc activation ($t_{(29)}$=0.394, p=0.696; data not shown). Both groups had equal numbers of Arc-positive cells during a single trial (SWIM; $t_{(25)}$=–0.32, p=0.75) and had greater Arc expression overall compared to rats that had been trained on the task (Figure 9d). All Arc-positive cells counted in this experiment were also NeuN-positive (data not shown), confirming Arc expression only in mature neurons.

3.3.2 Experiment 2

3.3.2.1 Neonatal treatment did not affect acquisition of a platform location on the MWM during extended training. Both treatment groups performed equally on a 48HR memory test.

Extensive training improved acquisition latency and memory for the platform. Neonatally-infected and control rats acquired the platform location during extended

training at the same rate and improved over days of training ($F_{(1,56)}$=31.0, p<0.001) (Figure 10a). Both groups of rats performed equally well at the 48HR memory probe test ($t_{(46)}$= 1.64, p=0.619) (Figure 10b).

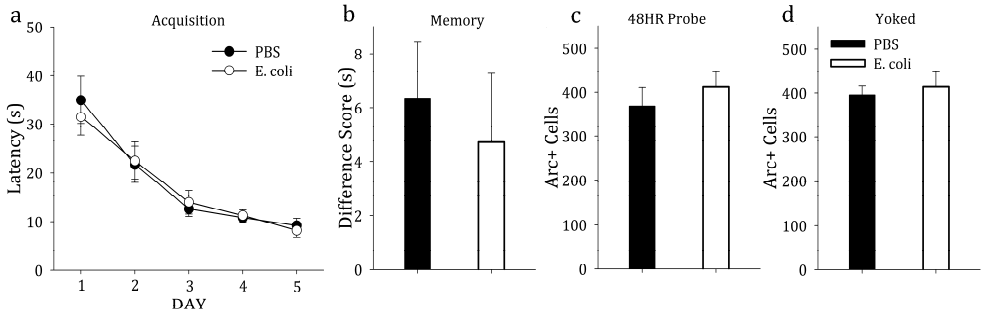

Figure 10: All rats exhibit similar acquisition, memory and neuronal activaton following extensive training. a) Both neonatally-infected rats and controls acquired the platform location at the same rate during extensive training (5 days of training with 10 trials per day) ($F_{(1,56)}$= 31.0, p<0.001). b) When tested 48HR after their last training trial, both groups performed equally well on the probe test ($t_{(14)}$ = 1.08, p=0.298). c) When the brains of the trained rats were assessed, both groups had similar numbers of Arc-positive cells in the DG ($t_{(14)}$ = -0.781, p=0.448). d) The "yoked" rats that matched the trained rats in latency in the pool had similar Arc-positive cell counts in the DG, regardless of neonatal treatment ($t_{(14)}$ = -0.44, p=0.67).

3.3.2.2 Both controls and neonatally-infected rats had similar levels of Arc activation in the DG during the 48HR probe following extended training.

Both groups also had equal Arc-positive cells in the DG during the 48HR memory test following extended training ($t_{(14)}$=−0.781, p=0.448) (Figure 10c) and the rats that were yoked to the extended training rats also showed equal Arc-positive cells in the DG ($t_{(14)}$=−0.44, p=0.67) (Figure 10d). Interestingly, in contrast to minimal training (Exp. 1), overall Arc expression in yoked rats did not differ between the trained and yoked groups (Figure 10, Panels c and d).

3.3.3 Experiment 3

3.3.3.1 Neonatal treatment did not affect platform location acquisition on extended training to one platform location (NW) or a subsequent reversal platform location (SE).

Reversal training after extensive training tested cognitive flexibility. Both groups acquired the platform location during the MWM training to both the original and reversal platform locations and improved over 5 and 3 days of training, respectively ($F_{(4,159)}$=142.4, p<0.001 and $F_{(2,95)}$=140.0, p<0.001, respectively) (Figure 11a).

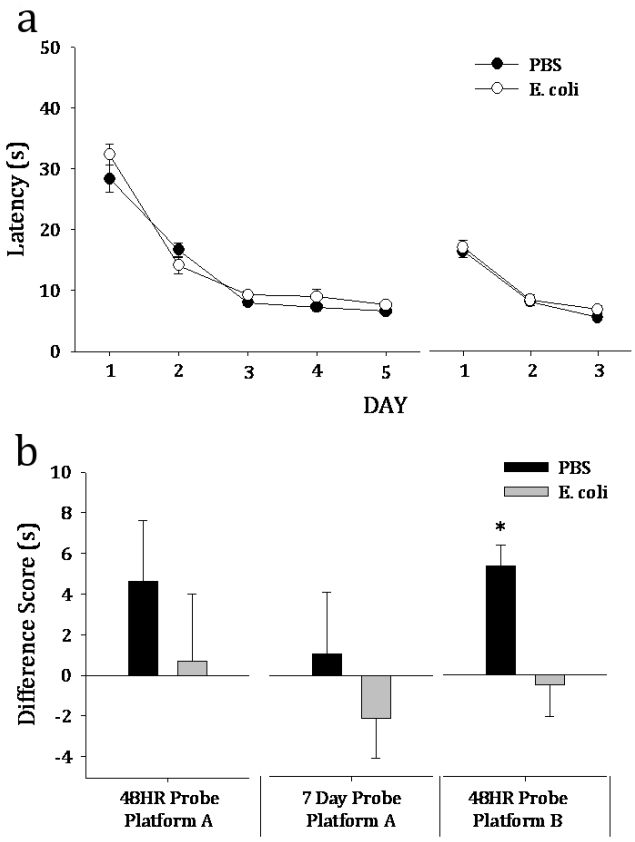

Figure 11: Neonatally-infected rats are significantly impaired on a reversal task, despite acquisition of both the original and reversal platforms comparable to controls. a) Both neonatal treatment groups acquired the platform at the same rate during the 5 days of extensive training ($F_{(4,159)} = 142.4$, $p<0.001$) and the 3 days of reversal training ($F_{(2,95)} = 140.0$, $p<0.001$). b) Controls and neonatally-infected rats performed similarly on the 48HR ($t_{(13)} = 0.78$, $p=0.451$) and 7 Day ($t_{(13)} = 0.33$, $p=0.745$) memory probe tests for the original platform. After reversal training, however, neonatally-infected rats had an impaired memory for the reversal platform location ($t_{(28)} = 2.98$, $p=0.006$).

3.3.3.2 Both groups performed equally on memory tests to the original platform location, but neonatally-infected rats demonstrated a memory impairment for the reversal platform location.

For the original platform location (NW), both groups of rats performed equally well at the 48HR memory probe test ($t_{(13)}$= 0.78, p=0.451) (Figure 11b, 48HR Platform A). Each group also performed equally on the 7 day memory probe test ($t_{(13)}$=0.33, p=0.745) (Figure 11b, 7 Day Platform A). However, the neonatally-infected rats had significantly impaired memory for the reversal platform location during the 48HR probe ($t_{(28)}$=2.98, p=0.006) (Figure 11b, 48HR Platform B).

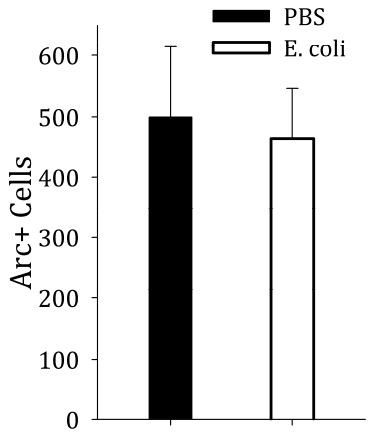

Figure 12: Neuronal activation does not differ between treatment groups at the 48HR reversal probe. In spite of a behavioral difference in which neonatally-infected rats performed worse on the probe test, both groups had equal numbers of Arc-positive cells in the DG during that probe ($t_{(14)}$ = -0.259, p=0.8).

3.3.3.3 Both treatment groups had similar Arc activation in the DG after the 48HR memory probe on the reversal platform location.

Both groups had equal counts of Arc-positive cells in the DG during the 48HR memory test for the reversal platform ($t_{(14)}$=–0.259, p=0.8) (Figure 12).

3.4 Discussion

We assessed the impact of neonatal infection on spatial learning and memory in adulthood, based on the growing evidence suggesting that early-life immune activation permanently alters the function of the hippocampus. Following minimal training, neonatally-infected adult rats acquired a place memory for the water maze platform more efficiently than controls, whereas no group differences in acquisition or memory probe performance were observed following extensive training. However, even after extensive training on the reversal task, neonatally-infected rats were markedly impaired on the 48HR memory test for the reversal platform. Finally, neuronal activation assessed using Arc protein was paradoxically reduced in neonatally-infected rats in response to the minimally-trained platform memory probe, during which performance was enhanced in this group, suggesting Arc expression within the DG may correlate with accuracy on certain cognitive tasks and perhaps predict flexibility on future performance.

The mRNA and protein level expressions of *Arc* have been analyzed in detail in the CA1 and CA3 regions of the hippocampus during spatial exploration tasks (4, 10, 12, 265, 267, 268). Arc activation in the CA1 and CA3 is highly correlated with place cell firing in the same regions during the same behavioral tasks (4, 9, 11), and Arc expression has become a valid correlate of cell activation in place of prior invasive *in vivo* studies or *in vitro* studies of the CA1 or CA3. In the DG, however, Arc expression has only recently

been explored. Arc expression in the DG identified newborn neurons that had matured and were capable of activation (269, 270). In another study, Arc mRNA and protein expression following two exploration bouts did not result in clear network activation in the DG (268). Recent work by the same group showed that renewed or continuous transcription within the same population of DG granule cells is responsible for sustained Arc expression up to 8H after an exploratory event (271). Our study is one of the first to examine Arc expression in the DG after a complex hippocampal-dependent task, assessing its correlation with memory retrieval after minimal and extensive water maze training paradigms.

Significantly reduced Arc expression in the DG coupled with improved memory in the neonatally-infected rats after minimal training was initially surprising. However, recent evidence demonstrates that irradiated mice with reduced neurogenesis have increased Arc expression in the DG during impaired performance on a hippocampal-dependent task (e.g. active place avoidance) (272), similar to our PBS controls. Another recent study observed sustained Arc mRNA expression in the DG for hours following exploration and suggested BDNF release as a mechanism for this continuous expression (271). We have previously reported that neonatally-infected rats have reduced hippocampal BDNF at baseline and following fear conditioning in adulthood (222); thus, enduring changes in BDNF signaling may contribute to altered Arc expression in the DG of neonatally-infected rats in response to initial spatial learning. Reduced Arc expression

during a challenging task, such as the memory test following minimal MWM training, may be correlated to increased accuracy on hippocampal-dependent tasks. The minimally trained accuracy seen in the neonatally-infected rats may, however, interfere with memory flexibility on the reversal task.

Alterations in neuronal circuitry may not be the primary cause for the memory differences we have observed between control and neonatally-infected rats. The MWM task is known to activate the hypothalamic-pituitary-adrenal (HPA) axis and to increase plasma corticosterone (CORT) levels (273). While we did not measure CORT during or after the MWM task, we have shown that neonatally-infected rats have attenuated serum CORT levels following stressors (e.g., inescapable tail shock or restraint stress), but are not different from controls at baseline (228). It is possible that attenuated CORT levels during the acquisition of the task could have contributed to improved performance after minimal training or altered neuronal activation in the DG, but we are unable to test CORT levels in the experiments described here. In addition to changes in CORT responses, neonatally-infected rats demonstrate increased microglial reactivity within the hippocampus following fear conditioning (108). Fear conditioning causes increased microglial reactivity after a single trial, but we have not examined microglial markers or inflammatory cytokines following several days of water maze training. Alterations in microglial signaling could underlie the changes in neuronal activation we observed in the hippocampi of these rats during memory testing. In addition to changes

in microglia, there may be changes at the receptor level on neurons (e.g., composition of NMDA receptor subunits on granule cells in the DG) that we are unable to measure here, but we have measured Arc protein in an attempt to assess potential differences at the level of hippocampal circuitry in the neonatally-infected rats.

Neonatal treatment had no effect on 48HR probe performance or DG Arc expression in extensively trained rats that were trained to only one platform location. To our surprise, the improved performance by neonatally-infected rats did not hold during the 48HR probe test for the reversal platform location. As we have seen previously, cognitive deficits in the neonatally-infected rats are often revealed following a second challenge in adulthood, including aging (221) and endotoxin exposure (108, 147-149). The cognitive challenge of reversal learning may have provided a similar "unmasking." The neonatally-infected rats had significantly worse memory performance than controls on the behavioral task, but both groups had similar Arc-positive cells in the DG. The similarity in Arc expression may be the result of many trials of exposure to the same environment; indeed, as we observed, both minimal and extensive training on the task resulted in lower induced Arc expression on average compared to rats that experience a single trial (SWIM). The equivalent protein expression between neonatal treatment groups in response to reversal memory testing could also be reflective of well-strengthened synapses that do not require the particular cellular mechanisms that Arc provides to maintain LTP or synaptic strength, especially after many exposures to the

same environment. Rats in Experiment 2 that were "yoked" by trial latencies to extensively trained rats had similar Arc expression in the DG compared to rats that learned, even without a platform as a goal. Thus, after many trials, the act of learning does not appear to mediate DG Arc expression further and the neural networks of the DG no longer correlate with behavior on spatial learning tasks.

The lack of differences in Arc expression in DG after reversal memory testing may be because the alterations in signaling occur in other brain regions, such as prefrontal cortex and striatum that are required for reversal task learning and memory. The hippocampus is critical for spatial learning, but the orbitofrontal region of the prefrontal cortex (PFC) has been implicated in reversal learning (274-276). Furthermore, lesions of the medial prefrontal cortex impair reversal platform location acquisition on a water maze task (277). In another water maze study, lesions of the medial striatum in adult rats significantly impaired acquisition and memory on a reversal task (278). We can explore the possibilities of neural signaling changes in these regions in future studies.

Future studies can also examine the specific effects of our neonatal infection model on these neural substrates. Others have shown that cortical circuitry is altered by prenatal and neonatal infection, including studies demonstrating that inflammatory signaling molecules inhibit dendrite development in cortical neurons *in vitro* (279) and apoptosis occurs in the prefrontal cortex, cerebellum and dentate gyrus for several

weeks post-inoculation with Bornavirus (280). Infection with an AIDS-like murine virus (LP-BM5) impaired reversal acquisition on the water maze as well (281), indicating that altered immune signaling within the brain may underlie deficits on the reversal task.

As we and others have shown, perinatal infection alters spatial learning in adulthood (e.g., 142, 144, 146) and may alter the underlying neural circuitry for spatial learning, but this hypothesis remains to be fully explored. In the hippocampus, a critical neural substrate for spatial learning, immune signaling molecules, such as cytokines and chemokines, have been implicated in synaptic formation and transmission (for review, see 282), offering a potential mechanism by which perinatal infection may have its effects on normal neural and synaptic function. Immune molecules are increasingly implicated in normal neural communication and any enduring changes in the hippocampal neural connectivity in neonatally-infected rats may underlie their distinct spatial learning behavior. While many of the cellular and molecular effects of neonatal infection remain to be elucidated, it seems that hippocampal function, especially on spatial learning tasks, is reliably altered by early-life immune activation. The examination of changes in neural circuitry are beginning to shed more light on the influence of the immune system within the nervous system and its effects on normal learning and memory, and the neonatal infection model provides a foundation with which we can explore the critical homeostatic functions for neuroimmune interactions in cognitive function. The enduring effects of such an infection demonstrate that cognitive

disabilities and disruptions may have their origins in the brain's earliest days and the assessment of such difficulties must evaluate the entire lifespan to elucidate underlying causes.

4. Environmental enrichment alters glial antigen expression and neuroimmune function in the adult hippocampus

4.1 Introduction

Environmental enrichment (EE) is a housing manipulation that increases physical and social stimuli and is often used to modulate hippocampal plasticity and to rehabilitate the injured brain. The impact of EE on improved neural outcomes following insult is often attributed to increased neurogenesis and/or survival. However, glia (microglia and astrocytes) are the primary immunocompetent cells of the brain, and their function is critical in both injury and repair. The functions of glia in repair may, in fact, be primary underlying mechanisms for improvement or rehabilitation observed after EE (210, 214, 218, 283).

We examined the impact of 7 weeks of daily EE in rats on neurogenesis and glial alterations within the hippocampus and cortex. We also assessed the cytokine and chemokine response within these regions to a peripheral immune challenge (lipopolysaccharide; LPS) at the end of the EE period. Notably, our enrichment protocol was applied *prior* to inflammatory insult (LPS), in contrast to the majority of studies that first induce injury and thereafter explore the capacity of EE to rescue cell viability or overall function. Our rationale for this approach was to explore the capacity of an environmental change to "buffer" the neuroinflammatory potential of the brain (i.e., prevention rather than rehabilitation). We report that EE specifically alters glial antigen

expression within the DG of the hippocampus, and markedly attenuates the immune response to peripheral LPS within the same region without significantly impacting measures of neurogenesis. These changes were specific to the hippocampus, as the glial, neuronal, and cytokine responses within the cortex remained unchanged by EE. Notably, the blunted immune response within the hippocampus of EE rats was specific to a subset of cytokines and chemokines, indicating that these animals were not immunocompromised or deficient. Taken together, the reduced expression of these particular immune molecules may lend insight into the neuroprotective phenotype of EE as well as the unique sensitivity of the hippocampus to inflammatory insult.

4.2 Materials and Methods

4.2.1 Animals and Environmental Enrichment

Thirty-two adult (P60) male Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN). All rats were pair-housed upon arrival and allowed 1 week to acclimate to the home cage environment. During the course of the experiment, all rats were maintained at 23°C on a 12:12-h light:dark cycle (lights on at 0700 EST) and given rodent chow and filtered drinking water ad libitum. After 1 week, 8 pairs were randomly assigned to environmental enrichment (EE) for a period of 7 weeks for 12 hours per day during their dark cycle, while 8 pairs remained in their home cages (HC). This shortened EE paradigm was selected to reflect a more realistic enrichment intervention, in which access to EE would be limited. EE rats were placed in the same

enrichment boxes each day at the same time each day. They were also removed from their enriched environments at the same time each day and returned to their home cages. The enrichment boxes (55.9 cm x 35.6 cm x 30.5 cm) contained quarter-inch corn-cob bedding (identical to home cage controls), a running wheel, a PVC tube and various small objects and toys. Enrichment and exercise may impact neurogenesis in the hippocampus differently (284); however, our aim was to maximize the effects on neurogenesis with both manipulations. The boxes were cleaned once weekly at the same time as home cage changes. All environments and conducted experiments were approved by the Duke University Institutional Animal Care and Use Committee.

4.2.2 Administration of BrdU

HC and EE rats were injected intraperitoneally (i.p.) with bromodeoxyuridine (BrdU) (Sigma-Aldrich; 150mg/kg), once daily for 3 days, beginning one week after the start of daily enrichment. This time point was chosen because previous literature demonstrates that key changes in neurogenesis and alterations in cell survival occur within the first week after plasticity-inducing conditions, such as EE or increased exercise (192, 285-287). Moreover, as mentioned previously, microglia play a key homeostatic role in neuronal survival during the first week after neurons are born in adulthood (105). Enrichment continued for 6 weeks after the first injection.

4.2.3 Adult Immune Challenge

After 7 weeks of differential housing and 2 hours prior to sacrifice, HC and EE rats were injected i.p. with either sterile saline (SAL) or a 100 μg/kg dose of bacterial lipopolysaccharide (LPS) suspended in sterile saline. These treatments resulted in the four groups shown in this study: HC injected with SAL (HC-SAL), HC injected with LPS (HC-LPS), EE injected with SAL (EE-SAL), and EE injected with LPS (EE-LPS).

4.2.4 Tissue Preparation

Two hours after the SAL or LPS injections, rats were deeply anesthetized with a ketamine/xylazine cocktail. Prior to the start of the perfusion, blood (2ml) was collected by cardiac puncture and stored on ice until centrifugation. Then rats were perfused transcardially with saline to clear brain vessels of blood. Their brains were extracted; one hemisphere was placed intact into 4% paraformaldehyde solution while the hippocampus and parietal cortex were rapidly dissected from the remaining hemisphere and immediately frozen via immersion in isopentane for later RNA extraction and protein analysis. Intact hemispheres were alternated between post-fixation and dissection and equally distributed across groups. Post-fixed hemispheres were submerged 2 days later in 30% sucrose plus 0.1% sodium azide for cryoprotection and were sliced following submersion. Using a -20°C cryostat, the intact hemispheres were sliced into forty um coronal sections, taking 5 series of 12 sections each. Sections were

stored at 4°C in a 0.1% sodium azide solution until free-floating immunohistochemistry was performed.

For quantitative real-time PCR, RNA was extracted from hippocampal and adjacent parietal cortex tissue using the Trizol method and DNAse-treated. Complementary DNA (cDNA) was synthesized from 100ng of isolated RNA for analysis of gene expression using the Qiagen QuantiTect Reverse Transcription Kit (Valencia, CA, USA) or from 500ng of isolated RNA using the RT² First Strand Kit (SABiosciences/Qiagen, Frederick, MD, USA).

4.2.5 Immunohistochemistry

4.2.5.1 Basic Immunohistochemistry (IHC) Protocol

Label-specific antibody differences are noted following this basic protocol.

Free-floating sections were first rinsed for 3x5 min in 0.01M phosphate buffered saline (PBS) and also rinsed before each subsequent step, except between the blocking and primary antibody steps. Next, sections were washed in 50% methanol for 30 min. Sections were then quenched in 0.6% hydrogen peroxide for 30 min and then blocked for another 30 min in 5% normal goat serum and 0.3% Triton-X to block and permeabilize, respectively, in PBS (blocking buffer). Sections were then incubated overnight at room temperature in primary antibody in blocking buffer. The following day, sections were incubated for 2 h at room temperature in a solution of biotinylated secondary antibody in blocking buffer. The Avidin-Biotin Complex (ABC) method was used to bind a

complex of streptavidin-biotin peroxidase to the secondary antibody (1 h incubation), which was then developed with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) for 2-15 min to produce a colorimetric stain. Sections were mounted on gel-coated slides, dehydrated and coverslipped with Permount.

4.2.5.1.1 BrdU

One series of coronal sections were stained with BrdU to quantify all newly dividing cells in the granule cell layer of the dentate gyrus in the hippocampus at the time of BrdU injections. BrdU IHC is similar to the method described above, but includes denaturing steps, which follow the hydrogen peroxide quenching step. Following quenching, sections were incubated at 65°C in 50:50 formamide:saline-sodium citrate (SSC) solution for 2 h and then rinsed for 2x5 min in 2X SSC. Next, sections were incubated in 2N HCl at 37°C in a water bath for 30 min followed by a 0.1M boric acid wash. The sections were then placed into blocking buffer and then the primary antibody buffer overnight (1:300 mouse monoclonal BrdU, Roche Diagnostics, Indianapolis, IN, USA). The next day for secondary antibody incubation, sections were incubated in biotinylated anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA. USA).

4.2.5.1.2 DCX

One series of coronal sections was stained with doublecortin (DCX) to identify immature neurons. Normal horse serum replaced normal goat serum for this stain. In the primary antibody step, sections were incubated with DCX antibody (1:200, goat

polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA). For the secondary antibody step, sections were incubated in biotinylated anti-goat antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

4.2.5.1.3 GFAP

One series of coronal sections was stained with glial fibrillary acidic protein (GFAP) to identify astrocytes. In the primary antibody step, sections were incubated with GFAP antibody (1:10,000, mouse monoclonal, Chemicon International, Temecula, CA, USA). For the secondary antibody step, sections were incubated in biotinylated anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

4.2.5.1.4 Iba1

One series of coronal sections was stained with ionized calcium binding adaptor molecule 1 (Iba1) to identify microglia. For the primary antibody step, sections were incubated in Iba1 antibody (1:10,000, rabbit polyclonal, Wako Pure Chemical Industries, Ltd). In the secondary antibody step, sections were incubated in biotinylated anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

4.2.6 Quantification

4.2.6.1 Cell Quantification

Quantification of BrdU-positive cells in the granule cell layer of the dentate gyrus in the hippocampus was performed using a Nikon Eclipse 80i microscope on a Dell PC running StereoInvestigator software (MBF Bioscience, Inc., Williston, VT, USA). The

boundaries of the dentate gyrus were traced using this software at a magnification of 10X and BrdU+ cells in the region of interest were counted at 40X. Five sections were counted for each rat and each section was at least 200 micrometers from the previously counted section. The counts from each individual section were combined to create a single BrdU+ cell count value per rat. Further, BrdU+ counts for rats in the same Housing-Treatment group were averaged to obtain a single BrdU average per group.

4.2.6.2 Densitometry

Quantification of GFAP-positive and Iba1-positive cells was performed using ScionImage densitometry. Seven sections per rat were analyzed for the dentate gyrus, CA1, and CA3 sub-regions of the hippocampus as well as the parietal cortex (Figure 1A). Digitized images of each region of interest (ROI) were taken at 20X using a Nikon Eclipse 80i microscope and digital camera on a Dell PC running PictureFrame software. Each region was traced using ScionImage after converting the image to grey scale. Signal pixels of an ROI were defined as having a grey value of 3 SDs above the mean grey value of a cell-poor area close to or within the ROI. The number of pixels and the average grey values above the set background were then computed for each ROI and multiplied by the area of the traced ROI, resulting in an integrated area density measurement. All values for each of the 7 sections per rat were averaged to obtain a single integrated density value per sub-region of interest for each rat. A two-way ANOVA was used to analyze the effects of Housing and Immune Challenge on the

individual values obtained for each rat. Integrated density values for rats in the same Housing-Treatment group were averaged to obtain a mean integrated density value per group that is shown on the graphs in Figure 15.

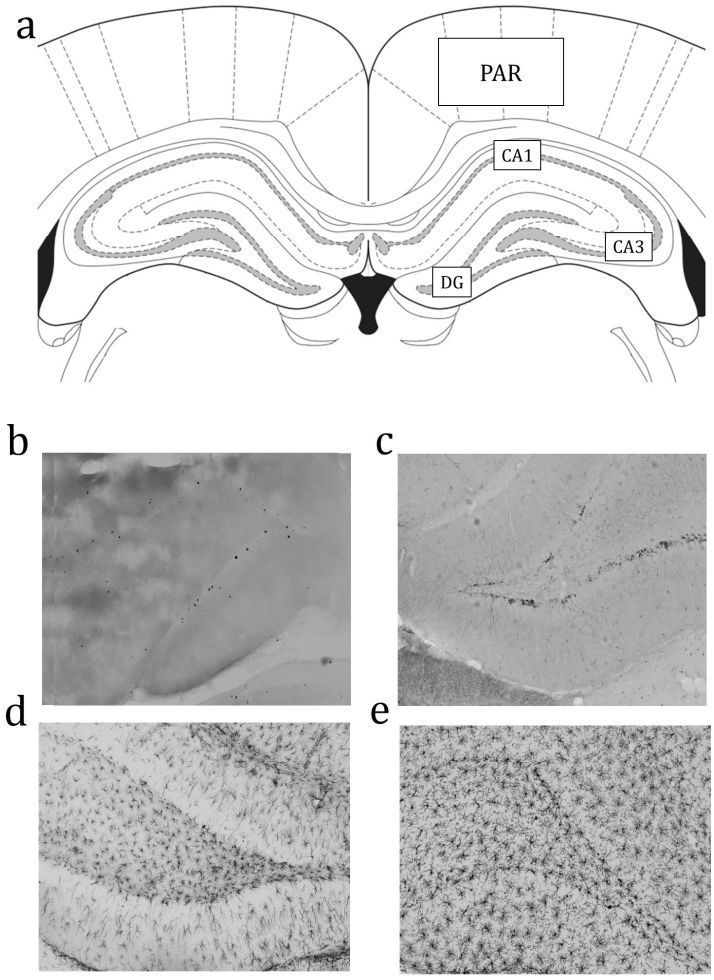

Figure 13: Regions analyzed for density measures and representative images from immunohistochemical staining. All representative photomicrographs are from the DG of a randomly selected EE rat. a) Regions of interest for density analysis. b) bromodeoxyuridine (BrdU), c) doublecortin (DCX), d) glial fibrillary acidic protein (GFAP), and e) ionized calcium binding adaptor molecule 1 (Iba1). Scale bar = 100μm for images B-E.

4.2.6.3 Quantitative Real-Time PCR

Gene expression was measured using quantitative real-time PCR with primers designed to measure 85 rat inflammatory cytokines, chemokines and receptors (SABiosciences/Qiagen; Cat. No PARN-011) or primers listed below using the RT^2 SYBR® Green qPCR Master Mix (Cat. No PA-010, SABiosciences/Qiagen, Frederick, MD, USA) following the manufacturer's protocol. For the first method, pairs of rats from each treatment group were pooled into 3 samples for a final *n* of 3/treatment group. For the second method, 8 rats/group were analyzed.

4.2.6.3.1 qRT-PCR Analysis

Threshold amplification cycle number (C_t) was determined for each reaction within the linear phase of the amplification plot and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method (230, 231). Relative gene expression across groups was compared using a two-way ANOVA with housing (home cage or enrichment) and immune challenge (SAL or LPS) as factors. Significant interactions were followed with either the Holm-Sidak or Tukey's post-hoc test with an α-level of $p < 0.05$ to determine group differences.

4.2.6.3.2 Primer Specifications

Real-time quantitative PCR primers were obtained from SABiosciences/Qiagen from Cat No. PARN-011 or designed by our laboratory and purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequences: *GAPDH:* Fwd –

GTTTGTGATGGGTGTGAACC, Rev – TCTTCTGAGTGGCAGTGATG; interleukin-1 receptor antagonist (*IL-1ra*): Fwd – GTCTGGAGATGACACCAAG, Rev – TCGGAGCGGATGAAGGTAA; glial derived neurotrophic factor (*GDNF*): Fwd – ATTCAAGCCACCATCAAAAG, Rev – TCAGTTCCTCCTTGGTTTCG; brain-derived neurotrophic factor (*BDNF, exon 5*): Fwd – ATCCCATGGGTTACACGAAGGAAG, Rev – AGTAAGGGCCCGAACATACGATTG.

4.2.7 Protein Assessment

4.2.7.1 Multiplex

A panel of 9 cytokines and chemokines (MCP-1, MIP-1α, IL-1β, IL-10, IFN-γ, IL-18, GRO/KC, Rantes, and TNFα) were measured in serum with a commercially available Luminex bead assay (Millipore Milliplex MAP Kit, Cat# RCYTO-80K). Blood from each rat was clotted and centrifuged after collection. The supernatant (serum) was then diluted 5-fold and analyzed according to the manufacturer's instructions. GRO/KC and Rantes were above the detectable range and IL-10 was below the detectable range following these methods; thus, these proteins were not reported.

4.2.7.2 Corticosterone Assessment

Total serum corticosterone concentrations were assessed in serum using a colorimetric EIA kit from Assay Designs, Inc. (Ann Arbor, MI). The assay was run according to the manufacturer's instructions except that serum (5 μl) was diluted 1:50 in

0.05% steroid displacement-modified assay buffer. The detection limit of the assay was 27 pg/ml, and the intra-assay coefficient of variation was 2.03%.

4.2.8 Statistical analyses

All of the analyses for this study were two-way ANOVAs comparing Housing (HC and EE) and Immune Challenge (SAL and LPS). *F* values for each analysis are reported in Section 3. Significant interactions were followed with either the Holm-Sidak or Tukey's *post hoc* comparison with an α-level of $p < 0.05$ to determine group differences.

4.3 Results

4.3.1 Environmental enrichment increases glial marker density within the DG, whereas markers of neurogenesis remain unchanged.

To assess the overall impact of EE on cell genesis/survival within the brain, we measured the expression of BrdU, which labels all newly dividing cells, using immunohistochemistry. The total number of BrdU+ cells within the DG was analyzed as a function of housing and LPS injection using a two-way ANOVA. Surprisingly, there was no effect of EE on total BrdU within the DG 6 weeks after BrdU administration (Figure 14a). Thus, we also assessed expression of the immature neuronal marker DCX in adjacent sections. DCX expression is independent of injection time point, a factor that may have contributed to the lack of change in BrdU by EE. However, there were also no significant changes in DCX within the DG following 7 weeks of EE (Figure 14b). As

expected, we did not observe significant BrdU or DCX staining outside the DG (not shown).

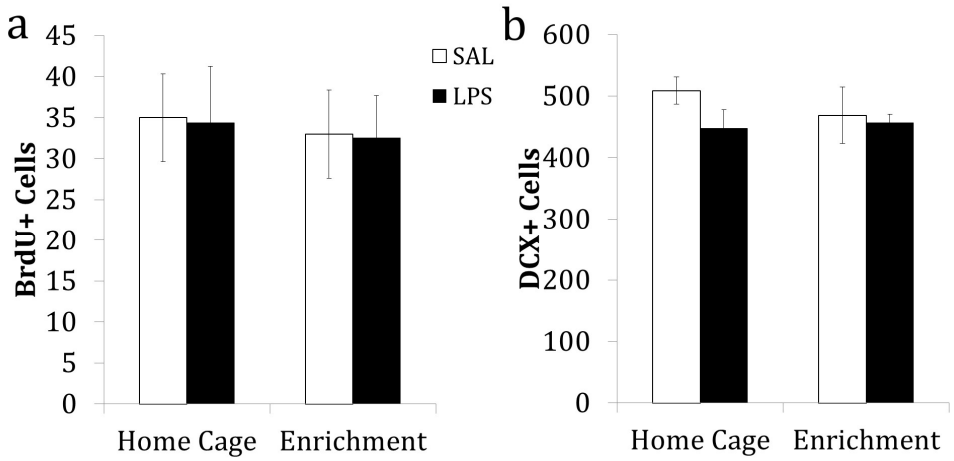

Figure 14: Daily environmental enrichment for 7 weeks does not increase cell survival in the dentate gyrus of the hippocampus. All rats (HC and EE) were given injections of BrdU one week after the beginning of the enrichment paradigm. Six weeks later, all rats were killed 2HR after an LPS injection. a) BrdU cell counts were not changed by EE and b) neither were DCX cell counts. Values are means ± SEMs of 8 rats/group.

To assess the impact of EE on glia, we assessed the expression of the glial antigens GFAP (for astrocytes) and Iba1 (for microglia). In contrast to measures of neurogenesis, there were increases in the density of both GFAP and Iba1 in the EE group. GFAP staining was increased by EE ($F_{(1,28)}$=23.2, p<0.001) only in the DG sub-region of the hippocampus and was not altered in either the CA1 or CA3 sub-regions (Figure 15a). Iba1 density in the DG was also significantly increased by EE ($F_{(1,28)}$=23.5, p<0.001), as well as by LPS treatment ($F_{(1,28)}$=4.45, p<0.05), an effect predominantly driven by the EE group (Figure 15c). Similar to GFAP, Iba1 density was not altered in either the

CA1 or CA3 sub-regions of the hippocampus. Furthermore, GFAP and Iba1 staining densities were not different in the hippocampal-adjacent parietal cortex across housing and treatment groups (Figure 15, b and d, respectively).

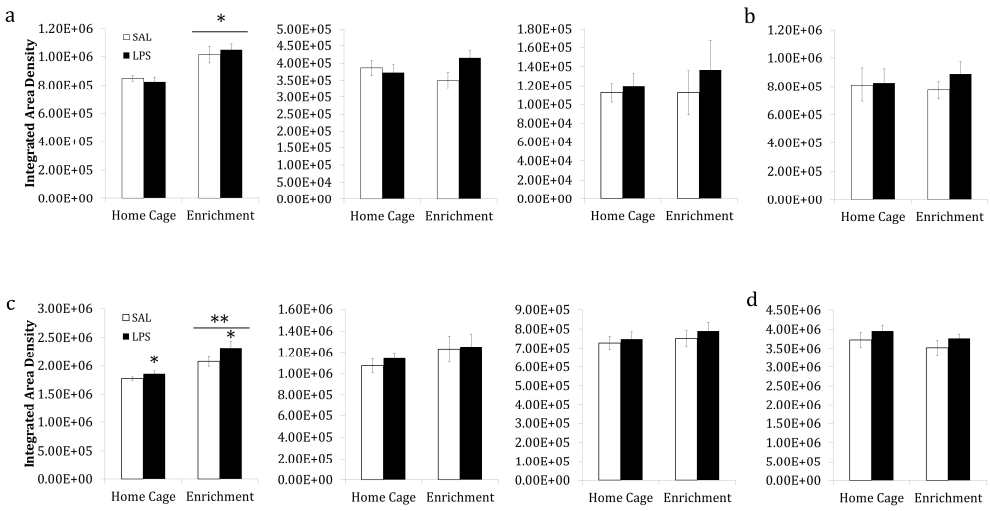

Figure 15: Environmental enrichment increases glial density in the dentate gyrus (DG), but not in the CA1 or CA3 of the hippocampus or the adjacent parietal cortex. a) The density of astrocyte marker GFAP (glial fibrillary acidic protein) was increased in the DG of EE rats and not in HC rats. In other sub-regions of the hippocampus, CA1 and CA3, GFAP density was similar between the two housing groups. b) Additionally GFAP density in the parietal cortex adjacent to the hippocampus was similar in each housing group. c) The density of microglial marker Iba1 was increased in the DG of EE rats compared to HC rats. Iba1 density in the DG was also increased following an LPS injection and that effect is driven by changes in EE rats. Microglial density was not altered in the CA1 or CA3 regions of the hippocampus (c) or the parietal cortex adjacent to the hippocampus (d). Values are means ± SEMs of 8 rats/group. * Significantly different from home cage, $p<0.05$. ** Significantly different from SAL, $p<0.05$.

4.3.2 Environmental enrichment increases BDNF within the hippocampus

We assessed the expression of two growth factors within the hippocampus, BDNF and GDNF, which are well described for their roles in neurogenesis and cellular

differentiation, especially during environmental enrichment (212-214, 288). Following 7 weeks of EE, BDNF mRNA was significantly increased within the hippocampus of EE rats ($F_{(1,28)}$=5.7, p<0.05) (Figure 16a). In contrast, GDNF mRNA was unaffected by housing (p>0.05) (Figure 16b). The expression of both growth factors was reduced by LPS (BDNF: $F_{(1,28)}$=8.8, p<0.01; GDNF: $F_{(1,28)}$=10.4, p<0.01).

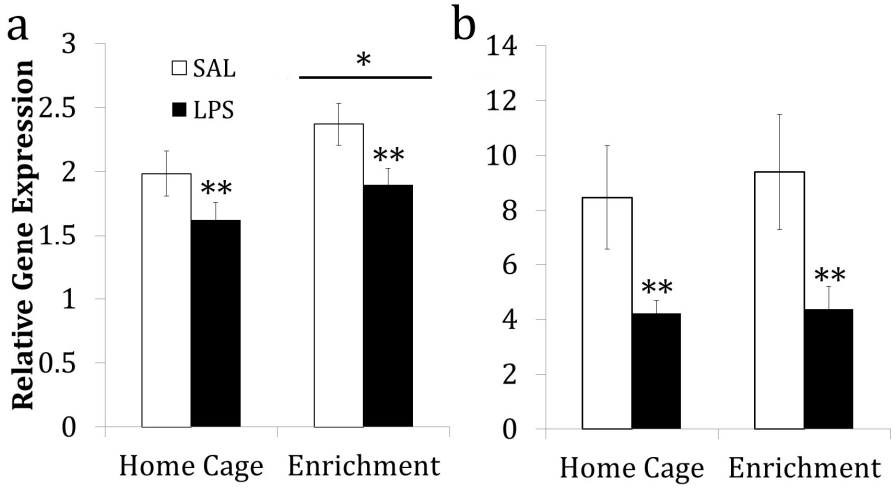

Figure 16: Environmental enrichment increased brain-derived neurotrophic factor (BDNF) mRNA expression (a), but not glial-derived neurotrophic factor (GDNF) mRNA expression (b). Both growth factors' expression was significantly reduced by LPS treatment. Values are means ± SEMs of 8 rats/group. * Significantly different from home cage, p<0.05. ** Significantly different from SAL, p<0.01.

4.3.3 Environmental enrichment attenuates the hippocampal response to LPS for a subset of cytokines and chemokines

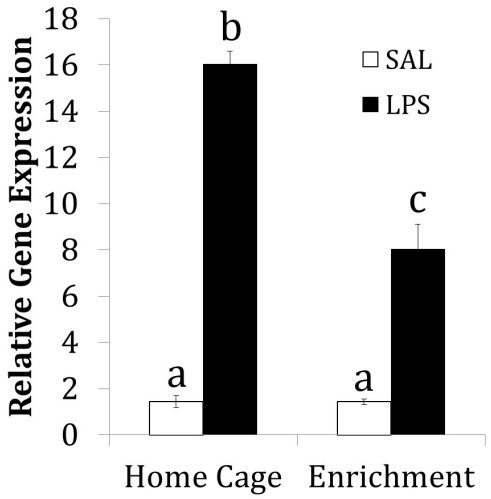

Figure 17: Environmental enrichment attenuates the interleukin-1β (IL-1β) response in the hippocampus following an LPS challenge. Rats from both housing groups were not different at baseline and responded to the LPS challenge. EE rats had a signficantly blunted increase in *IL-1β* mRNA expression following LPS compared to home cage rats. EE-LPS had significantly lower *IL-1β* mRNA expression compared to HC-LPS rats, $p<0.001$. Values based on means ± SEMs for 3 pairs (6 rats)/group. [a] Baseline value $p>0.05$. [b] Significantly increased from baseline, $p<0.05$. [c] Significantly incraesed from baseline and significantly lower than the HC rsponse, $p<0.05$.

To assess the impact of EE on the central immune response to a peripheral inflammatory challenge, we measured inflammatory cytokine and chemokine mRNA expression within the hippocampus and cortex 2 hours following LPS or saline. The pro-inflammatory cytokine interleukin-1β (*IL-1β*) increased within the hippocampus after LPS, but this increase was significantly attenuated in EE rats compared to HC ($F_{(1,8)}$=42.1, $p<0.001$) (Figure 17). Additionally, mRNA expression of several members of the tumor necrosis factor (TNF) pathway was reduced in EE-LPS rats compared to HC-LPS: TNF*α*

(Figure 18a) ($F_{(1,8)}$= 11.3, p<0.05), the receptor gene TNF receptor super family 1a (*Tnfrsf1a*, Figure 18b) ($F_{(1,8)}$=5.4, p<0.05), the receptor gene TNF receptor super family 1b (*Tnfrsf1b*, Figure 18c) ($F_{(1,8)}$=9.6, p<0.05), and lymphotoxin alpha (*Lta* or *TNFβ*, Figure 18d) ($F_{(1,8)}$=17.2, p<0.005). Finally, the expression of three chemokines known primarily for their functions in leukocyte and monocyte recruitment was reduced in the hippocampus in response to LPS in the EE compared to HC rats: monocyte chemotactic protein 1 (*MCP-1*/*Ccl2*) ($F_{(1,8)}$=9.4, p<0.05), macrophage inflammatory protein 1 alpha (*MIP-1α*/*Ccl3*) ($F_{(1,8)}$=42.1, p<0.001) and macrophage inflammatory protein 2 alpha (*MIP-2α* /*Cxcl2*) ($F_{(1,8)}$=10.7, p<0.05) (Figure 19). For all genes listed above with the exception of *Lta*, baseline mRNA expression did not differ between saline-treated HC and EE rats.

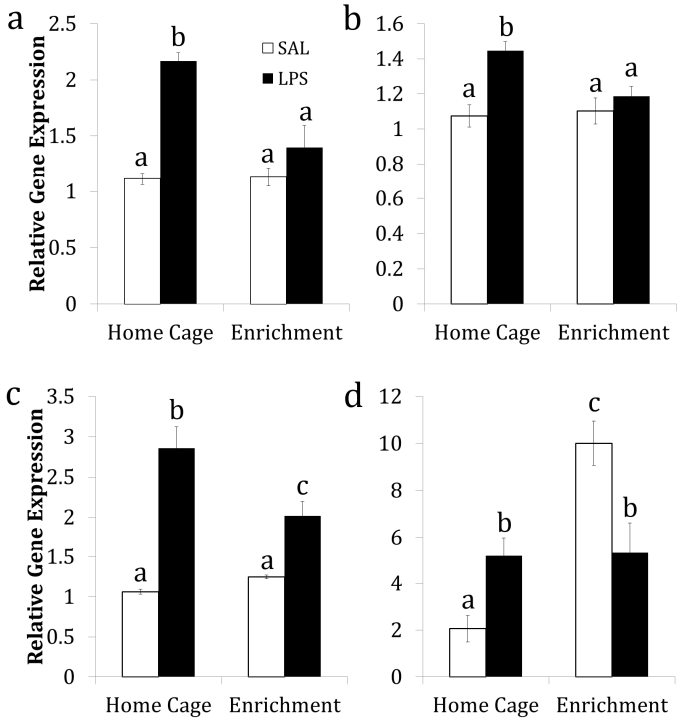

Figure 18: Environmental enrichment altered the expression of several members of the tumor necrosis factor (TNF) family following an LPS challenge. Tumor necrosis factor alpha (*TNFα*) (a), TNF receptor super family 1a (*Tnfrsf1a*) (b) and TNF receptor super family 1b (*Tnfrsf1b*) (c) had significantly blunted mRNA expression in response to an immune challenge in EE rats. Lymphotoxin alpha (*TNFβ*) (d) was increased at baseline and its expression was reduced in EE rats following LPS. Values based on means ± SEMs for 3 pairs (6 rats)/group. All differences, $p<0.05$.

Further assessment in the hippocampus revealed an extensive list of pro-inflammatory cytokine, chemokine and receptor genes whose transcription increased following LPS treatment (main effect of LPS treatment in 2-way ANOVAs; all p's<0.05) without alteration by housing (Table 1).

Table 1: Chemokines and cytokines in the hippcampus altered by treatment with LPS, regardless of housing condition. All *p*-values <0.05. Values based on means ± SEMs for 3 pairs (6 rats)/group.

Gene	Reference ID	HC-SAL	HC-LPS	EE-SAL	EE-LPS	Main Effect of LPS
Ccl22	NM_057203	1.6±0.3	4.4±1.2	1.2±0.1	5.4±0.3	$F_{1,8}$=32.2, p<0.001
Ccl7	NM_001007612	4.6±1.8	42.1±15.7	5.3±2.7	13.8±1.9	$F_{1,8}$=8.1, p<0.022
Cxcl1	NM_030845	4.2±0.4	82.0±20.1	2.6±0.8	41.5±4.0	$F_{1,8}$=32.4, p<0.001
Cxcl9	NM_145672	1.7±0.1	27.3±3.5	1.4±0.2	26.6±3.8	$F_{1,8}$=96.4, p<0.001
Cxcl10	NM_139089	6.1±0.7	1061.2±149.7	7.7±3.6	915.5±153.3	$F_{1,8}$=83.9, p<0.001
Cxcl11	NM_182952	2.5±0.3	603.4±148.3	3.1±1.4	381.9±59.8	$F_{1,8}$=37.5, p<0.001
IL-1a	NM_017019	3.1±0.4	1.7±0.4	3.5±0.1	1.7±0.4	$F_{1,8}$=19.5, p=0.002
IL1ra	NM_022194	2.8±0.4	6.4±1.5	3.3±0.8	6.5±1.8	$F_{1,28}$=10.4, p=0.003
IL2rg	NM_080889	1.3±0.2	7.9±0.9	1.3±0.1	6.13±0.2	$F_{1,8}$=145.9, p<0.001
IL6ra	NM_017020	1.3±0.1	2.4±0.1	1.2±0.1	2.0±0.2	$F_{1,8}$=52.1, p<0.001
IL8rb	NM_017183	2.3±0.8	9.2±4.6	1.6±0.3	7.4±1.9	$F_{1,8}$=6.1, p=0.039
IL-15	NM_013129	1.5±0.1	1.3±0.2	1.6±0.1	1.1±0.1	$F_{1,8}$=5.7, p=0.044

In contrast to the hippocampus, all gene expression changes within the parietal cortex adjacent to the hippocampus were due to LPS treatment alone (main effect of LPS treatment in 2-way ANOVAs; all *p*'s<0.05), without an effect of housing (Table 2). Many

of the same genes were upregulated following LPS treatment in both the hippocampus and parietal cortex.

Table 2: Chemokines and cytokines in the parietal cortex altered by treatment with LPS, regardless of housing condition. All *p*-values <0.05. Values based on means ± SEMs for 3 pairs (6 rats)/group.

Gene	Reference ID	HC-SAL	HC-LPS	EE-SAL	EE-LPS	Main Effect of LPS
Bcl6	XM_221333	1.1±0.1	1.6±0.1	1.2±0.02	1.9±0.2	$F_{1,8}$=39.5, p<0.001
Ccl3	NM_013025	1.4±0.2	4.9±0.5	2.1±0.4	4.2±0.3	$F_{1,8}$=62.7, p<0.001
Ccl5	NM_031116	1.4±0.3	2.4±0.5	1.6±0.2	2.5±0.4	$F_{1,8}$=6.0, p=0.040
Cxcl1	NM_030845	1.3±0.3	10.9±3.2	1.3±0.02	10.5±0.8	$F_{1,8}$=32.1, p<0.001
Cxcl2	NM_053647	1.8±0.2	62.8±20.2	1.8±0.7	40.0±6.2	$F_{1,8}$=21.9, p=0.002
Cxcl9	NM_145672	1.6±0.3	91.0±31.1	2.3±0.3	81.8±9.6	$F_{1,8}$=26.8, p<0.001
Cxcl10	NM_139089	2.0±0.7	208.8±50.0	1.7±0.3	205.8±13.5	$F_{1,8}$=63.0, p<0.001
Cxcl11	NM_182952	3.4±0.9	549.3±233.8	1.7±0.5	405.2±49.1	$F_{1,8}$=15.8, p=0.004
Cxcr5	NM_053303	1.2±0.3	3.8±1.0	1.4±0.2	3.5±0.7	$F_{1,8}$=13.5, p=0.006
IL-1b	NM_031512	2.6±0.4	29.5±10.3	2.3±0.8	19.4±3.7	$F_{1,8}$=15.9, p=0.004
IL1r2	NM_053953	1.4±0.4	3.0±0.6	1.7±0.2	2.2±0.1	$F_{1,8}$=8.4, p=0.02
IL2rg	NM_080889	1.5±0.2	7.2±1.0	1.1±0.04	6.1±0.4	$F_{1,8}$=95.0, p<0.001
IL6ra	NM_017020	1.1±0.04	2.6±0.3	1.2±0.03	2.2±0.2	$F_{1,8}$=44.2, p<0.001
IL8ra	NM_019310	3.0±0.9	6.6±1.1	2.2±0.8	7.0±0.6	$F_{1,8}$=23.5, p=0.001
IL8rb	NM_017183	2.1±0.8	7.5±1.8	1.5±0.3	6.4±1.3	$F_{1,8}$=18.9,

						p=0.002
IL-11	NM_133519	1.3±0.2	2.3±0.3	1.5±0.1	1.9±0.3	$F_{1,8}$=9.7, p=0.014
IL-15	NM_013129	1.6±0.03	1.2±0.1	2.0±0.1	1.3±0.2	$F_{1,8}$=13.7, p=0.006
Lta	NM_080769	2.1±0.5	3.4±0.4	1.6±0.6	2.6±0.4	$F_{1,8}$=5.5, p=0.046
Tnfrsf1b	NM_130426	1.3±0.2	2.9±0.4	1.3±0.1	2.4±0.1	$F_{1,8}$=43.5, p<0.001

Many of the same genes were upregulated following LPS treatment in both the hippocampus and parietal cortex.

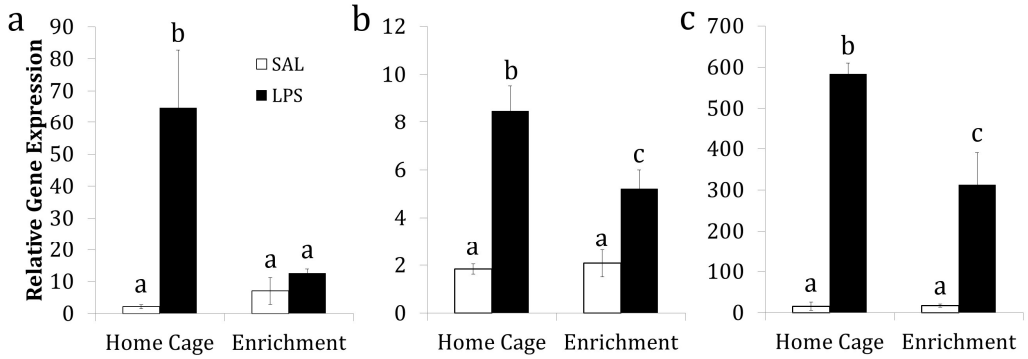

Figure 19: Following an LPS challenge, environmental enrichment altered the expression of several chemokines – monocyte chemotactic protein 1 (*MCP-1*/*Ccl2*) (a), macrophage inflammatory protein 1 alpha (*MIP-1α*/*Ccl3*) (b), and macrophage inflammatory protein 2 alpha (*MIP-2α*/*Cxcl2*) (c). All 3 chemokines had significantly blunted mRNA expression in EE-LPS rats compared to HC-LPS rats, $p<0.05$. [a] Baseline value, $p<0.05$. [b] Significantly increased from baseline (a), $p<0.05$. [c] Significantly increased from baseline and significantly lower than HC response. Values based on means ± SEMs for 3 pairs (6 rats)/group.

4.3.4 Environmental enrichment does not impact the peripheral immune or corticosterone response to LPS

We assessed a panel of 9 inflammatory cytokine and chemokine proteins within the serum using a Luminex bead-based assay, to determine whether the impact of EE on

the immune response to LPS was specific to brain. Each of the proteins assessed was also measured at the mRNA level within the brain (as discussed previously), though we were only able to measure a small subset of proteins in serum due to the limited availability of rat-specific analytes for the Luminex technology. We did not use Luminex to assess protein expression within *brain* tissue, as this technology has not been validated for rat brain and in our hands generated many high false positives (e.g., on the upper end of detection) without a significant LPS effect (data not shown). In short, we do not believe the measurements are accurate.

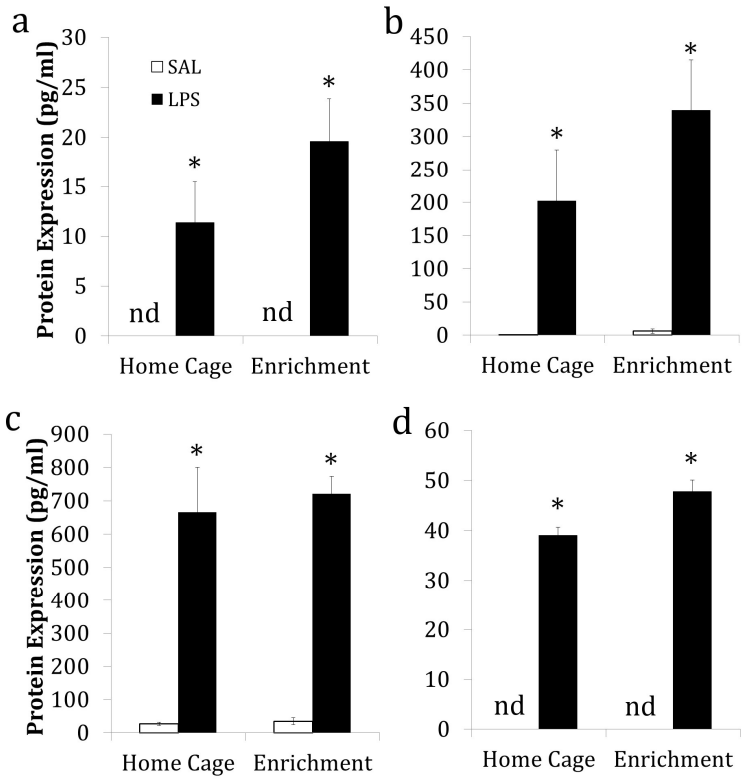

Figure 20: In the periphery, LPS treatment significantly increased protein expression in the serum of the following cytokines and chemokines: interleukin-1β (IL-1β) (a), tumor necrosis factor alpha (TNFα) (b), monocyte chemotactic protein 1 (MCP-1/CCL2) (c), and macrophage inflammatory protein 1 alpha (MIP-1α/CCL3) (d). Values are means ± SEMs of 8 rats/group. * Significantly different than SAL, $p<0.005$.

Our protein assessments in serum showed only an effect of LPS for each protein without an effect of housing. IL-1β (Figure 20a, $p<0.001$), TNFα (Figure 20b, $p<0.005$), MCP-1 (Ccl2) (Figure 20c, $p<0.001$) and MIP1α (Ccl3) (Figure 20d, $p<0.001$) were equally increased in the periphery, regardless of housing condition, unlike their responses within the hippocampus. Serum corticosterone, which can markedly impact pro-

inflammatory cytokine expression, was also increased by LPS treatment in all rats, without an effect of housing condition ($F_{(1,28)}$=276.3, p<0.001) (Figure 21).

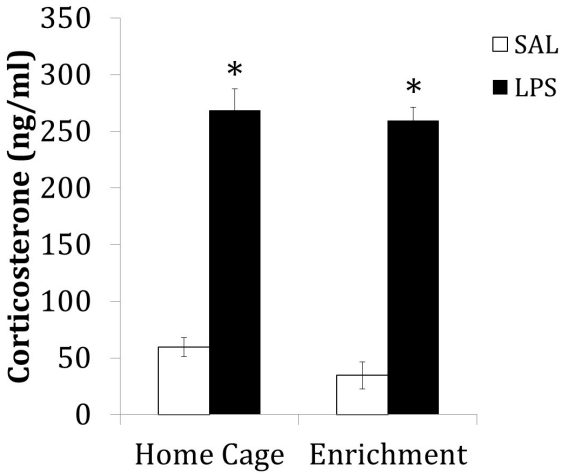

Figure 21: An immune challenge with LPS significantly increased serum corticosterone, regardless of housing group. Baseline corticosterone was not altered by housing. Values are means ± SEMs of 8 rats/group. * Significantly different from SAL, p<0.001.

4.3.5 Environmental enrichment increases CD200 expression, but does not alter the expression of CD200R, fractalkine (CX3CL1) and fractalkine receptor (CX3CR1).

To assess the effects of EE on neuron-glia interactions, we measured mRNA expression from the same hippocampal tissue assessed above in Figures 16-19. Neurons have direct contact with microglia and can alter their reactivity (234, 235). We did not measure any group differences in fractalkine (CX3CL1) expression or in the expression of its receptor (CX3CR1).

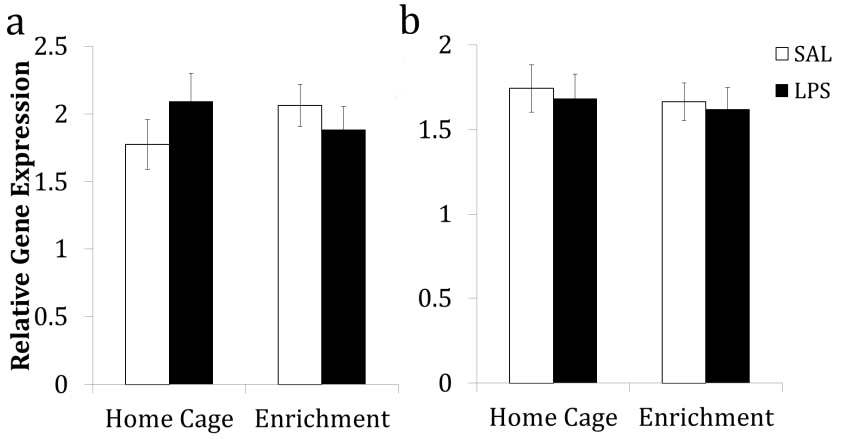

Figure 22: Fractalkine signaling is not altered by environmental enrichment. a) Fractalkine (CX3CL1) mRNA expression is not different between housing groups or treatment conditions. b) Fractalkine receptor (CX3CR1) expression is not altered in any treatment groups.

CD200 expression was not altered by housing or LPS treatment, but expression of its receptor, CD200R, was significantly increased in the hippocampi of EE rats ($F_{(1,28)}$=6.64, p=0.016).

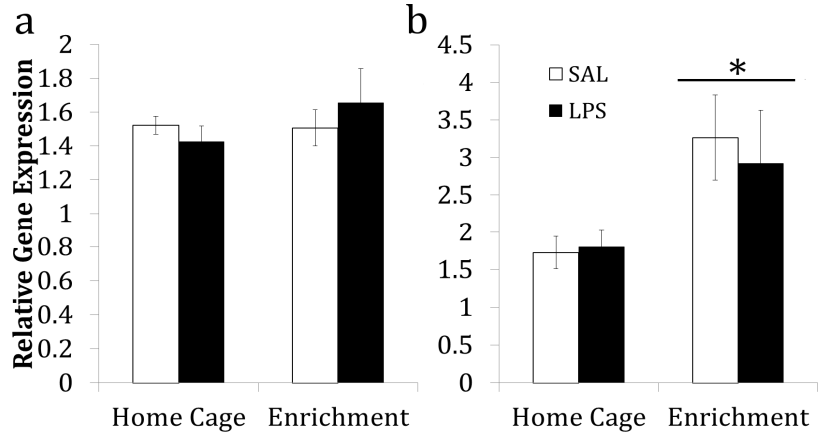

Figure 23: Environmental enrichment alters expression of CD200 receptor. a) CD200 mRNA expression is not different between housing and treatment groups. b)

CD200 receptor (CD200R) expression is increased in EE rats. * Significantly different from home cage (p<0.05).

4.4 Discussion

The hippocampus has long been considered to have increased plastic potential compared to other cortical regions, but with its increased plasticity may come augmented vulnerability. Cytokine receptors are distributed throughout the brain, but have one of the highest densities in the hippocampus (54, 289). The hippocampus also exhibits marked vulnerability (e.g., cell death and neural dysfunction) relative to other brain regions in response to diverse threats to homeostasis, including stress, epilepsy, stroke, and cardiac arrest (290-293). Thus the hippocampus may be particularly sensitive to immune-related alterations (294, 295). The adjacent parietal cortex that lies dorsal to the hippocampus is responsive to LPS (108); however, the neocortex and hippocampus differ dramatically in structure, connectivity and function. The finding that EE did not affect the neuroimmune response in the parietal cortex, whereas it had a dramatic effect in the hippocampus, underscores the distinction of the hippocampus from other cortical structures.

Environmental enrichment is a well-established protocol for altering the neurogenic niche of the subgranular zone (SGZ) of the DG of the hippocampus. We hypothesized that EE, which induces neuroprotection in a number of studies (214, 217, 296, 297), would attenuate the neuroinflammatory response to a peripheral immune challenge. Finally, we predicted that EE would impact not only neurogenesis but glia as

well, as shown in limited studies (209, 211, 298). Glia are the likely source of the neuroinflammatory cytokines and chemokines of interest, and the impact of EE could potentially alter these signaling molecules. We observed a markedly reduced pro-inflammatory phenotype within the brains of EE rats compared to controls, evidenced by the selective attenuation of a subset of cytokines and chemokines following peripheral LPS. This attenuation was restricted to the hippocampus, and coincident with increased expression of the glial antigens Iba1 and GFAP. Surprisingly, however, these changes were independent of increased neurogenesis, which we did not observe when assessed using BrdU given after 1 week of EE, or with the immature neuronal marker DCX after 7 weeks of EE.

Interestingly, neurogenesis itself is not required for cognitive improvements following enrichment (as shown by 299). Our enrichment paradigm was similar in duration by total days to many other studies (for meta-analysis, see 300), but few, if any, other studies alter housing *solely* during the dark cycle as we did in this study to more accurately model a "real-world" intervention. It is possible that this paradigm is more stressful than full-time housing; however, we did not observe any significant differences in corticosterone between home cage and EE rats. It is also possible that extending our enrichment paradigm to either full time housing or 10 to 12 weeks of limited daily enrichment would increase the population of immature neurons measured by DCX staining, based on prior findings in the field. Our paradigm did, however, elicit other

enduring changes in the hippocampi of EE rats. EE increased BDNF mRNA overall within the hippocampus, despite the acute suppression in response to LPS, which are both consistent with the literature (154, 213, 301, 302). Most strikingly, EE increased the density of both Iba1 and GFAP expression specifically within the DG, consistent with a previous report that wheel running exercise increases astrogliogenesis (209). Our densitometry assessments do not allow conclusions regarding gliogenesis or number of cells; however, the lack of change in total BrdU strongly argues against an increase in the number of new glia in EE rats. The data instead suggest that EE changes the *phenotype* of glia, altering their activation and attenuating their pro-inflammatory response to peripheral LPS, although this remains to be directly tested. Interestingly, the blunted neuroinflammatory response within the DG of EE rats occurring concomitant with the *increase* in classical glial "activation" markers runs counter to our initial prediction. However, we believe these data simply highlight the fact that little is known about the function of these markers. Moreover, there is a growing literature that distinguishes classical versus alternative activation states in microglia, the latter of which is associated more strongly with repair (73, 164). Thus, it is possible that EE shifts microglia into an alternatively activated phenotype, an intriguing possibility that we are currently exploring. Regardless of whether glial number or phenotype is influenced, the notable finding is that there is clearly an impact of EE on glia that occurs without any discernible change in newborn neurons. Importantly, glia were not changed in the CA1 or CA3

sub-regions of the hippocampus, nor were they altered in the adjacent parietal cortex, suggesting that the microenvironment of the DG is conducive to glial changes which may occur independent from neurogenesis. Indeed, glial plasticity may directly interact with and alter neuronal plasticity, questions that remain to be explored.

The immune response within the hippocampi of EE rats was markedly attenuated for a subset of cytokines and chemokines measured in our study. Importantly, not all measured immune molecules were blunted in the hippocampi of EE rats. Furthermore, the immune response was similar for each housing group in the parietal cortex as well as in the periphery. Within the hippocampus, however, EE rats had an attenuated response of interleukin-1β (IL-1β), the TNF family of genes, and several chemokines involved in the recruitment of leukocytes and monocytes. These families of genes indicate an altered hippocampal milieu in EE rats that may be less pro-inflammatory, more neuroprotective and less permeable to peripheral infiltrating immune cells.

As several studies, including our own, have shown, IL-1β is a pro-inflammatory molecule that is also important for normal brain function (52, 57, 154, 303-305). Importantly, physiological levels of IL-1β expression are critical for normal learning and memory (57), whereas levels that are either too low or too high become detrimental (108). In the present study, EE rats have a blunted IL-1β response to an LPS dose that elicits a robust increase in mRNA expression in HC controls. Numerous other studies

have demonstrated improved learning and memory performance in EE rats (299, 300, 306-310). Notably, the source of IL-1β during hippocampal-dependent learning, as well as during an immune response within the hippocampus, is solely CD11b+ microglia (108). Altered microglial reactivity in response to EE may therefore have significant consequences for learning and memory processes, especially in the context of infection or illness, which remains to be explored. Similarly, many studies use EE as a rehabilitation paradigm in a diseased brain model (e.g., Parkinson's disease, (296)); in these studies, IL-1β tone may exceed optimal levels in the inflamed, diseased brains without an EE intervention.

Tumor necrosis factor alpha (TNFα) is well characterized for its roles in inflammation and host defense, sepsis and, most intriguing for this study, apoptosis cascades (for review, see 311). The observed attenuation after an immune challenge of TNFα and several associated genes in EE rats compared to HC controls indicates a potential enduring change in the hippocampal microenvironment of enriched rats, such that one mechanism by which EE may increase neuroprotection following insults to the CNS (214, 217, 296) is via altered TNF tone and function, increasing the likelihood of cell survival by reducing apoptotic signaling. In addition to attenuated IL-1β and TNF responses, EE rats showed blunted responses for several chemokines known to influence the recruitment of circulating monocytes and leukocytes to the CNS. Monocyte chemoattractant protein-1 (MCP-1/CCL2) is important for recruitment of peripheral

monocytes into the brain during an immune challenge or infection (e.g. HIV; (312)). Following status epilepticus (SE) in rats, triggered by stimulation of the amygdala, a paradigm known to increase aberrant neurogenesis and neuronal injury in the hippocampus, macrophage inflammatory protein 1-alpha (MIP-1α/CCL3) is increased for up to 30 days (313). Taken together, the attenuated expression of these cytokines and chemokines in EE rats treated with LPS demonstrate possible mechanisms for neuroprotective and increased plasticity outcomes associated with EE.

In addition to attenuated expression of pro-inflammatory cytokines and chemokines, there was a change in CD200 receptor, which mediates interactions between neurons and microglia. While fractalkine (CX3CL1) and its receptor (CX3CR1) were not altered by EE, CD200R expression was increased in EE rats, regardless of immune challenge. Greater expression of CD200R, which potentially increases the sensitivity of microglia to neural signals via CD200, is the converse of our previous finding in the neonatally infected rats following fear conditioning (Chapter 2 and (108)). The increase in CD200R could be one mechanism by which microglial reactivity is attenuated by EE, though the mechanisms that cause this increase are yet unknown.

In summary, environmental enrichment is a relatively simple manipulation that results in robust beneficial outcomes for the brain. While previous studies have shown a role in post-insult rehabilitation for EE, our study provides evidence that enrichment need not follow the insult in order to be beneficial. Indeed, neuroinflammatory disease

states might be attenuated or delayed in their onset in the face of ongoing EE. The translational reach of this manipulation remains to be explored, but in animal models of neuroinflammation, EE may provide a simple preventative measure for negative outcomes. Furthermore, this study presents further evidence that EE alters microglia and astrocytes within the DG of the hippocampus. Though the mechanism remains to be investigated, these changes in glia in the DG may underlie the attenuated immune response in the brains of EE rats treated with LPS, and these same glial changes may be directly related to neural changes often associated with EE. As an increasing number of studies explore the effects of glia (both microglia and astrocytes) on normal brain function, manipulations that alter both glia and neurons alike may provide perspective on interactions between these cell types as well as their specific roles in the healthy brain.

5. Environmental enrichment alters the neurogenic niche: altered microglial reactivity requires interactions with other neural cell types

5.1 Introduction

The previous work on environmental enrichment (Chapter 4) demonstrated that EE prior to immune challenge increases glial antigen density (e.g., GFAP, Iba1) within the dentate gyrus, increases growth factors within the hippocampus and decreases pro-inflammatory signaling within the hippocampus at the transcription level following the peripheral immune challenge. While compelling, these data did not reveal which cell types were most affected by EE. This study sought to test the hypothesis that hippocampal microglia are altered by EE, which leads to the changes in Iba1 expression and/or changed microglial cell number as well as the attenuated cytokine and chemokine response within the hippocampus. Furthermore, the effects seen on astrocytes in the earlier EE experiment did not address changes in cell number in that population, which this study aimed to address.

In order to assess microglial function, they were isolated from other cell types in the hippocampus and treated *ex vivo* with an immune activator, lipopolysaccharide (LPS). LPS binds directly to toll-like receptor 4 (TLR4) on microglia and elicits a pro-inflammatory cascade of cytokines and chemokines. We predicted that a phenotypic change in microglia would be demonstrated by an attenuated secretion of inflammatory cytokines and chemokines, similar to the pattern we observed in the previous EE

experiment. In addition to the cell culture *ex vivo* manipulation, we used the other half of the brain to assess cell number of both microglia (Iba1+) and astrocytes (GFAP+), using immunohistochemical methods. With microglia, we could measure soma size in addition to cell number to determine any morphological alterations caused by EE without the influence of a peripheral immune challenge. These experiments would allow us to fully characterize the effects of EE on the glial cells of the hippocampus and provide potential mechanisms by which our previous results might occur.

5.2 Materials and Methods

5.2.1 Animals and Environmental Enrichment

Twenty-four adult (P60) male Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN). All rats were pair-housed upon arrival and allowed 1 week to acclimate to the home cage environment. During the course of the experiment, all rats were maintained at 23°C on a 12:12-h light:dark cycle (lights on at 0700 EST) and given rodent chow and filtered drinking water ad libitum. After 1 week, 6 pairs were randomly assigned to environmental enrichment (EE) for a period of 7 weeks for 12 hours per day during their dark cycle, while 6 pairs remained in their home cages (HC). This shortened EE paradigm was selected to mimic the housing of Experiment 4 as a realistic intervention and to assess the effects of this type of enrichment on other outcomes. EE rats were placed in the same enrichment boxes each day at the same time each day. They were also removed from their enriched environments at the same time

each day and returned to their home cages. The enrichment boxes (55.9 cm x 35.6 cm x 30.5 cm) contained quarter-inch corn-cob bedding (identical to home cage controls), a running wheel, a PVC tube and various small objects and toys. Enrichment and exercise may impact neurogenesis in the hippocampus differently (284); however, our aim was to maximize the effects on neurogenesis with both manipulations. The boxes were cleaned once weekly at the same time as home cage changes. All environments and conducted experiments were approved by the Duke University Institutional Animal Care and Use Committee.

5.2.2 Tissue Preparation

On the last day of enrichment, rats were deeply anesthetized with a ketamine/xylazine cocktail. Then rats were perfused transcardially with saline to clear brain vessels of blood. Their brains were extracted; one hemisphere was placed intact into 4% paraformaldehyde solution while the hippocampus and parietal cortex were rapidly dissected from the remaining hemisphere. Each dissected brain region was placed into an individual well in 2ml HBSS (Hanks Balanced Salt Solution; w/o Mg^{+2} and Ca^{+2}; Invitrogen) in a 6-well plate and taken for microglial isolation (see **5.2.3**). Intact hemispheres were alternated between post-fixation and dissection and equally distributed across groups. Post-fixed hemispheres were submerged 2 days later in 30% sucrose plus 0.1% sodium azide for cryoprotection and were sliced following submersion. Using a -20°C cryostat, the intact hemispheres were sliced into 40 um

coronal sections, taking 5 series of 60 to 70 sections each. Sections were stored at 4°C in a 0.1% sodium azide solution until free-floating immunohistochemistry was performed.

5.2.3. Microglia Isolation

Isolated hippocampi and parietal cortex regions were sliced into small pieces using a sterile razor and transferred into a 2 ml sterile tube with 1 ml of HBSS (w/o) (Invitrogen), which was spun at 300 X *g* at room temperature for 2 min. The tissue was then brought to a single-cell suspension using Miltenyi's Neural Tissue Dissociation Kit (P) (Miltenyi Biotec) in MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 7.2) for 15 min at 4°C. The tissue was passed through a 70 µm Nitex nylon filter and resuspended in 10 ml of HBSS (w/ Mg^{+2} and Ca^{+2}; Invitrogen). The tissue was then centrifuged at 300 X *g* at 4°C for 10 min. After the supernatant was aspirated, the tissue was resuspended in 30% Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) and centrifuged at 700 X *g* at 4°C for 10 min. The remaining pellet (demyelinated cells) were washed with 5 ml HBSS (w/o) and centrifuged at 300 X *g* at 4°C for 10 min. The demyelinated cells were further fractionated based on CD11b expression as follows (**5.2.3.1)**.

5.2.3.1 MACS enrichment of CD11b+ cells

Cells were resuspended in 250 µl of MACS buffer, stained with 2.5 µl of PE-conjugated mouse anti-rat CD11b/c beads (BD Biosciences Pharmingen) and incubated for 10 min in the dark at 4°C. The cells were washed with 1 ml of MACS buffer and

centrifuged at 300 X *g* at 4°C for 10 min. Cells were then incubated in 200 µl MACS buffer with 50 µl anti-PE MicroBeads (Miltenyi Biotec) at 4°C for 10 min. The suspension was washed with 5 ml of MACS buffer and centrifuged at 300 X *g* at 4°C for 10 min, and the supernatant was aspirated off. The cells were resuspended in 500 µl of MACS buffer and were passed through a nylon mesh filter onto magnetized LS columns (Miltenyi Biotec). The columns were washed with 4 ml of MACS buffer and the flow through was collected into a clean 15 ml conical (as CD11b/c-negative fraction). Magnetically labeled CD11b/c-positive cells were flushed out of the columns twice with MACS buffer into a clean 15 ml conical. Magnetically enriched cell fractions were centrifuged at 300 X *g* at 4°C for 10 min, followed by resuspension of CD11b+ cells in high-glucose (4.5 g/L) DMEM. Isolated CD11b+ cells were then placed into culture as follows (see **5.2.3.2**). Isolated CD11b/c-negative cells were resuspended in 500 µl PBS and centrifuged at 300 X *g* at 4°C for 5 minutes. After aspiration, CD11b/c-negative cell pellets were flash-frozen at -80°C until processing for RT-PCR.

5.2.3.2 *Ex vivo* LPS stimulation

Isolated CD11b+ cells were suspended in Neuro Media (high-glucose (4.5 g/L) DMEM supplemented with 1% Pen-Strep (GIBCO, Life Technologies, Grand Island, NY), 2mM L-glutamine (GIBCO), 1mM sodium pyruvate (GIBCO), 1% N2 supplement (GIBCO), 10% fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific, Waltham, MA) and 5 µg/ml forskolin (Sigma-Aldrich, St. Louis, MO)). Cells were incubated with

LPS (10 ng/ml) or media alone for 4 h at 37°C, 5% CO_2. For each condition, samples were run in duplicate. At the end of incubation, the plate was centrifuged at 1000 X *g* for 10 min at 4°C to pellet cells. Supernatant for each sample was collected and frozen at -20°C. The cell pellet was resuspended in 300 µl PBS, centrifuged at 350 X *g* for 10 min at 4°C, and the resulting pellet was flash frozen at -80°C until processing for RT-PCR.

5.2.4 Immunohistochemistry

5.2.4.1 Basic Immunohistochemistry (IHC) Protocol

Label-specific antibody differences are noted following this basic protocol.

Free-floating sections were first rinsed for 3x5 min in 0.01M phosphate buffered saline (PBS) and also rinsed before each subsequent step, except between the blocking and primary antibody steps. Next, sections were washed in 50% methanol for 30 min. Sections were then quenched in 0.6% hydrogen peroxide for 30 min and then blocked for another 30 min in 5% normal goat serum and 0.3% Triton-X to block and permeabilize, respectively, in PBS (blocking buffer). Sections were then incubated overnight at room temperature in primary antibody in blocking buffer. The following day, sections were incubated for 2 h at room temperature in a solution of biotinylated secondary antibody in blocking buffer. The Avidin-Biotin Complex (ABC) method was used to bind a complex of streptavidin-biotin peroxidase to the secondary antibody (1 h incubation), which was then developed with diaminobenzidine (DAB, Vector Laboratories,

Burlingame, CA, USA) for 2-15 min to produce a colorimetric stain. Sections were mounted on gel-coated slides, dehydrated and coverslipped with Permount.

5.2.4.1.1 GFAP

One series of coronal sections was stained with glial fibrillary acidic protein (GFAP) to identify astrocytes. In the primary antibody step, sections were incubated with GFAP antibody (1:10,000, mouse monoclonal, Chemicon International, Temecula, CA, USA). For the secondary antibody step, sections were incubated in biotinylated anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

5.2.4.1.2 Iba1

One series of coronal sections was stained with ionized calcium binding adaptor molecule 1 (Iba1) to identify microglia. For the primary antibody step, sections were incubated in Iba1 antibody (1:10,000, rabbit polyclonal, Wako Pure Chemical Industries, Ltd). In the secondary antibody step, sections were incubated in biotinylated anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

5.2.5 Cell Quantification

Quantification of Iba1-positive cells in the dentate gyrus in the hippocampus was performed using a Nikon Eclipse 80i microscope on a Dell PC running StereoInvestigator software (MBF Bioscience, Inc., Williston, VT, USA). The boundaries of the dentate gyrus were traced using this software at a magnification of 4X and Iba1+ cells in the region of interest were counted at 100X (oil). Five sections were counted for

each rat and each section was at least 200 micrometers from the previously counted section. Unbiased stereology was used to estimate the total numbers of Iba1+ cells. Cell numbers were estimated using the Cavalieri principle and the optical dissector method, with a 2mm guard zone. Microglial volumes were estimated using 5 rays of independent isotropic probes within the "Nucleator" function of the StereoInvestigator software. Estimated cell counts were calculated by the Mean Measured Section Thickness multiplied by total counts in 5 representative sections and the section periodicity (5) within StereoInvestigator to create a single Iba1+ estimated cell count per rat. Estimated volumes were calculated based on measured section volumes and section periodicity (5) to create a single estimated volume value per rat. The volumes of individually marked cells in the "Nucleator" function were averaged within a section and then averaged across sections for a single average cell volume measurement for each rat.

5.2.6 Quantitative Real-Time PCR

Gene expression was measured using quantitative real-time PCR with primers listed below using the RT^2 SYBR® Green qPCR Master Mix (Cat. No PA-010, SABiosciences/Qiagen, Frederick, MD, USA) following the manufacturer's protocol. The CD11b-negative population from the hippocampi of 12 rats/group was analyzed.

5.2.6.1 RNA Isolation, cDNA Synthesis and RT-PCR

Total RNA was isolated based on the TRIzol method (229). To increase mRNA yield from isolated cell samples, glycogen (Invitrogen, Life Technologies, Grand Island,

NY) was added at the isopropanol step. Following RNA isolation and enrichment, samples were DNase-treated, and cDNA was synthesized using the reverse transcriptase kit from QIAGEN. Amplification of cDNA was performed using a QuantiFast SYBR Green PCR kit (QIAGEN) on a Mastercycler ep *realplex* (Eppendorf). cDNA (1.5 μg in 1 μl) was added to a reaction master mix (12 μl) containing HotStarTaq Plus DNA Polymerase, QuantiFast Buffer, SYBR Green I, Passive reference dye, and gene-specific primers (500 nM each of forward and reverse primer).

5.2.6.2 qRT-PCR Analysis

Threshold amplification cycle number (C_t) (number of cycles to reach threshold of detection) was determined for each reaction within the linear phase of the amplification plot and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method (230, 231). Relative gene expression across groups was compared using a one-way ANOVA with housing (home cage or enrichment) as the factor.

5.2.6.3 Primer Specifications

Real-time quantitative PCR primers were designed by our laboratory and purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequences: *GAPDH:* Fwd – GTTTGTGATGGGTGTGAACC, Rev – TCTTCTGAGTGGCAGTGATG; *CD200:* Fwd – TGTTCCGCTGATTGTTGGC, Rev – ATGGACACATTACGGTTGCC; *CX3CL1:* Fwd – TCCAGGGCTGTCCCCGCAAA, Rev - ACAGGCAGGCAAGCAGGCAG

5.2.7 Multiplex

A panel of 9 cytokines and chemokines (CCL2/MCP-1, CCL3/MIP-1α, CXCL2/MIP-2α, CXCL5/LIX, IL-1α, IL-1β, IL-10, IL-18, and TNFα) were measured in the supernatant from the *ex-vivo* cell culture procedure with a commercially available Luminex bead assay (Millipore Milliplex MAP Kit, Cat# RCYTMAG-65K). The supernatant was analyzed according to the manufacturer's instructions. CXCL5 was below the detectable range following these methods; thus, this protein was not reported.

5.2.8 Statistical analyses

Analyses for immunohistochemistry were one-way ANOVAs comparing Housing (HC and EE). Analyses for multiplex were three-way ANOVAs with brain region (HP vs. PAR) housing (home cage vs. enrichment) and culture treatment (media vs. LPS) as factors followed by two-way ANOVAs based on interactions. *F* values for each analysis are reported in Section 5.3.

5.3 Results

5.3.1. Environmental enrichment reduces microglia volumes in the dentate gyrus compared to controls, but does not alter the number of microglia.

In previous work on environmental enrichment (Chapter 4), we assessed the expression of Iba1 with densitometry, a measure that does not determine numbers of cells or changes in antigen expression at the level of single cells (314). Based on the increase in Iba1 density, we performed unbiased stereology in conjunction with the

Nucleator function in the dentate gyrus to determine cell number and individual cell volumes. Estimated cell counts of Iba1+ cells were not different between enriched and home cage rats (Figure 22a; $F_{(1,22)}$=0.03, p=0.86). Estimated total region volumes calculated during stereology were not different between housing groups (Figure 22b; $F_{(1,22)}$=0.72, p=0.41). However, average cell volume as calculated by the Nucleator function (soma size X factor of section thickness) was reduced in the DG regions of enriched rats compared to home cage controls (Figure 22c; $F_{(1,22)}$=4.35, p=0.048).

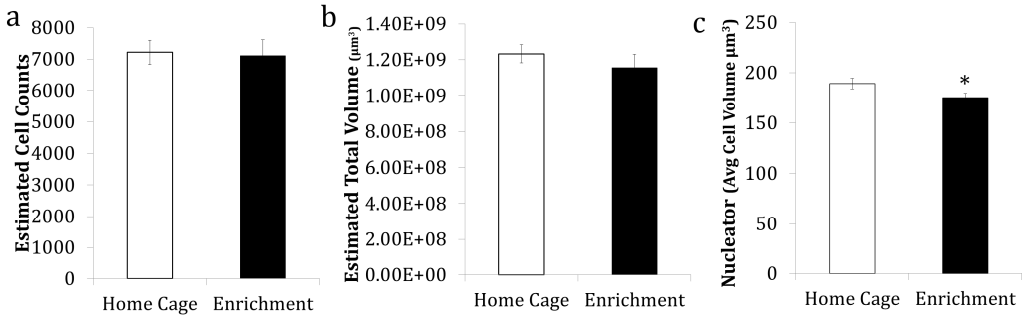

Figure 24: Microglial soma size is reduced in the DG of enriched rats, but total cell counts are not altered. a) Home cage and enriched rats have the same number of Iba1+ cells within the DG. b) Both housing conditions have similar DG volumes. c) Microglia volume is reduced in the DG of enriched rats, but not home cage controls. *Significantly less than Home Cage, p<0.05

5.3.2. Environmental enrichment does not alter the number of GFAP+ astrocytes within the dentate gyrus.

Similar to Iba1 antigen expression in Chapter 4, GFAP density was increased in the DG of enriched rats. Based on this increase, we performed unbiased stereology in the dentate gyrus to determine cell numbers of GFAP+ cells. Estimated cell counts of GFAP+ cells were not different between enriched and home cage rats (Figure 23a; $F_{(1,22)}$

=0.18, p=0.68). Estimated total region volumes calculated during stereology were not different between housing groups (Figure 23b; $F_{(1,22)}$= 0.01, p=0.92).

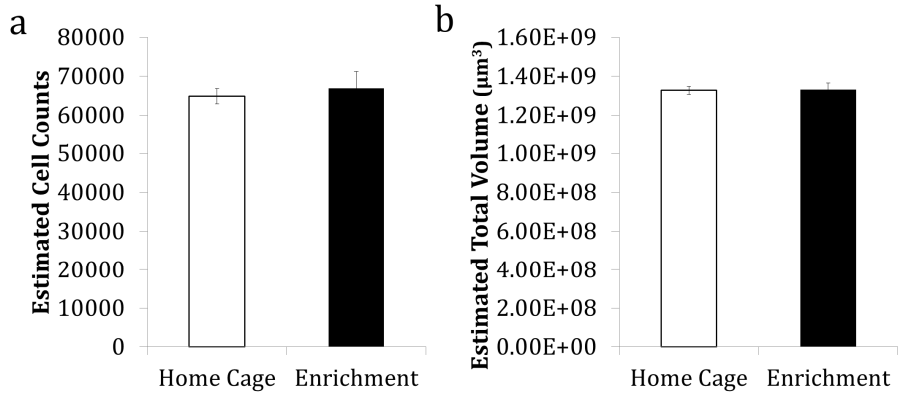

Figure 25: Total GFAP+ cell counts are not different between housing groups. a) Home cage and enriched rats have the same number of GFAP+ cells within the DG. b) Both housing conditions have similar DG volumes.

5.3.3. Isolated hippocampal and parietal cortical microglia from enriched rats do not differ in their cytokine response to *ex-vivo* LPS stimulation in culture.

We assessed a panel of 9 inflammatory cytokine and chemokine proteins within the cell culture supernatant using a Luminex bead-based assay, to determine whether the impact of EE altered the intrinsic reactivity of microglia within the hippocampus and adjacent parietal cortex. Our protein assessments in supernatant showed only an effect of LPS for each protein without an effect of housing. In hippocampal microglia, IL-1β (Figure 24a; F(1,44) = 15.36 p=0.0003) and IL-10 (Figure 25a; F(1,44)=15, p=0.0004) were equally increased in response to LPS, regardless of housing condition.

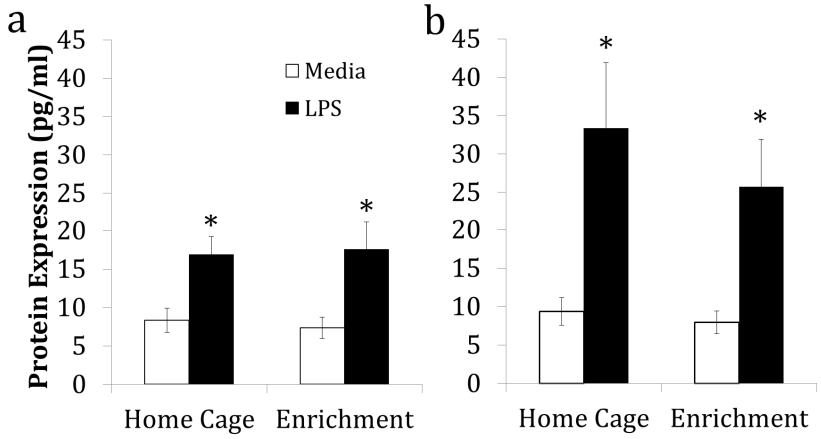

Figure 26: Isolated microglia from home cage and enriched rats had similar IL-1β responses to LPS in culture. a) IL-1β expression is increased in isolated hippocampal microglia exposed to LPS in culture, regardless of housing condition. b) IL-1β expression is increased in isolated microglia from the parietal cortex that were exposed to LPS in culture, regardless of housing condition. *Significantly greater than Media, $p<0.05$

Similarly in microglia isolated from the parietal cortex, IL-1β (Figure 24b; $F(1,44) = 14.9$ $p=0.0004$) and IL-10 (Figure 25b, $F(1,44)=23.9$, $p<0.0001$) were equally increased in response to LPS in both home cage and enriched rats.

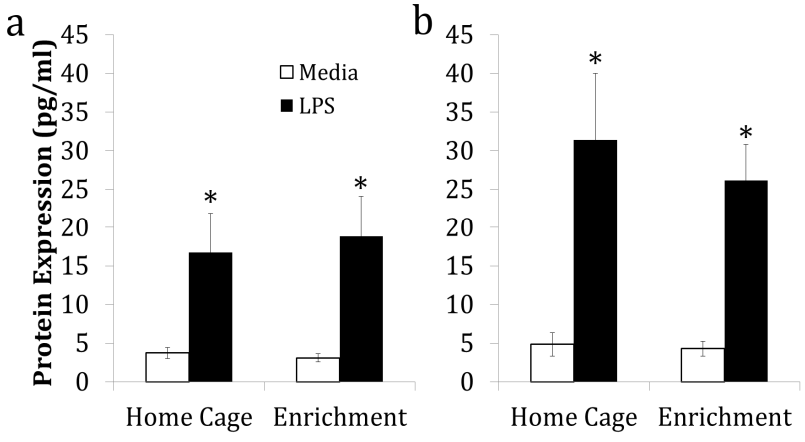

Figure 27: Isolated microglia from home cage and enriched rats had similar IL-10 responses to LPS in culture. a) IL-10 expression is increased in isolated

hippocampal microglia exposed to LPS in culture, regardless of housing condition. b) IL-10 expression is increased in isolated microglia from the parietal cortex that were exposed to LPS in culture, regardless of housing condition. *Significantly greater than Media, $p<0.05$

In addition to changes in IL-1β and IL-10, IL-1α, MIP-1α (CCL3), MIP-2α (CXCL2), and TNFα expression was increased in hippocampal following LPS treatment (main effect of LPS treatment in 2-way ANOVAs; all p's<0.05) without alteration by housing (Table 3). There were no significant differences in expression of IL-18 and MCP-1 (CCL2) between culture treatment or between housing groups (p>0.05).

Table 3: Chemokines and cytokines in CD11b+ isolated from the hippocampus altered by treatment with LPS with no effect of housing condition. All p-values<0.05. Values based on means ± SEMs for 12 rats/group.

Protein	HC-Media	HC-LPS	EE-Media	EE-LPS	Main Effect of LPS
IL-1α	6.6±1.6	28.7±2.8	4.2±0.4	31.5±6.4	$F_{1,44}$=47.6, p<0.001
MIP-1α	156.5±37.1	310.5±30.8	121.6±21.1	273.6±35.2	$F_{1,44}$=23.4, p<0.001
MIP-2α	292.4±62.2	1212.3±105.4	190.3±32.4	995.5±134.9	$F_{1,44}$=86.9, p<0.001
TNFα	3.4±0.7	41.9±5.3	2.7±0.6	36.4±5.3	$F_{1,44}$=92.4, p<0.001

The same pattern of expression in response to LPS occurred in microglia isolated from the parietal cortex (Table 4). Notably, the cytokine response from microglia in the parietal cortex was significantly greater than hippocampal microglia for several proteins: IL-1α ($F_{(1,88)}$=4.16, p=0.044), IL-1β ($F_{(1,88)}$=4.93, p=0.029), IL-10 ($F_{(1,88)}$=3.87, p=0.05), MIP-1α ($F_{(1,88)}$=8.33, p=0.004) and MIP-2α ($F_{(1,88)}$=7.34, p=0.008).

Table 4: Chemokines and cytokines in CD11b+ cells isolated from the parietal cortex altered by treatment with LPS with no effect of housing condition. All *p*-values<0.05. Values based on means ± SEMs for 12 rats/group.

Protein	HC-Media	HC-LPS	EE-Media	EE-LPS	Main Effect of LPS
IL-1α	7.0±1.5	47.1±8.8	5.9±0.8	41.5±9.5	$F_{1,44}$=33.2, p<0.001
MIP-1α	154.2±16.1	394.1±44.8	169.8±17.4	421.4±51.6	$F_{1,44}$=46.2, p<0.001
MIP-2α	346.7±41.7	1591.5±132.9	302.9±29.0	1552.2±161.4	$F_{1,44}$=134.3, p<0.001
TNFα	3.1±0.5	44.6±5.6	2.8±0.4	48.6±5.8	$F_{1,44}$=115.8, p<0.001

5.3.4. The CD11b/c-negative population does not differ in mRNA expression of signaling molecules that interact with microglia

The CD11b/c-negative population was analyzed for mRNA expression of genes related to neuronal interaction with microglia. As noted previously (Chapters 2 & 4), neurons can make direct contact with microglia with their surface-expressed and secreted products (234, 235). In the CD11b/c-negative population from the hippocampus, neither CX3CL1 mRNA expression (Figure 26a; $t_{(22)}$ = -0.33, p=0.74) nor CD200 mRNA expression (Figure 26b; $t_{(22)}$ = 0.48, p=0.64) is different between the two housing groups.

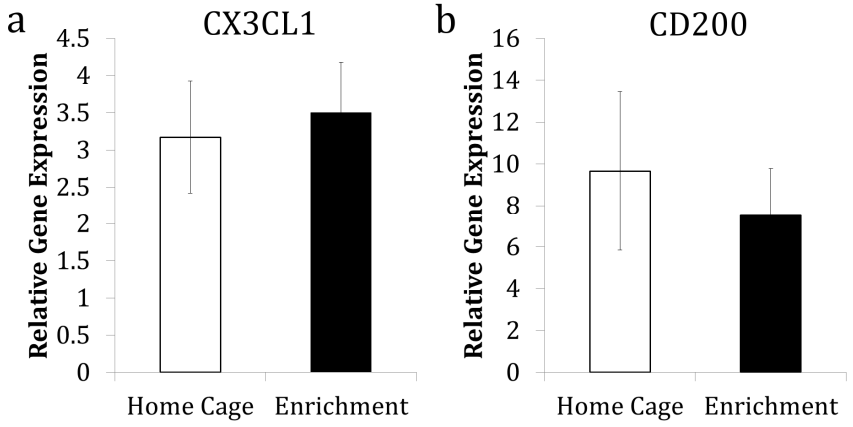

Figure 28: Neuronal molecules that interact directly with microglia are not different between housing conditions in the hippocampal CD11b/c-negative population. a) Fractalkine (CX3CL1) is similarly expressed at the mRNA level in CD11b/c-negative cells. b) CD200 mRNA expression is similar in both housing groups in the CD11b/c-negative population.

These results replicate the lack of difference between housing groups seen in the previous EE experiment (Chapter 4), indicating that neural signals to microglia are not changed by EE, but rather microglial sensitivity to neural modulation is likely augmented following EE (i.e., increased CD200R expression).

5.4. Discussion

We have demonstrated that environmental enrichment has a marked effect on glial antigen expression in the DG, increasing GFAP expression and Iba1 expression, though the latter was significantly increased by peripheral immune challenge as well. In this study, we have shown that the number of GFAP+ cells is not altered in the DG of EE rats compared to HC controls. The estimated hippocampal volume represented in these counts is not different between the two groups; therefore, we can conclude that the

number of cells per cubic micron (μm^3) is similar within the DG of both housing groups. Iba1 counts showed a similar pattern of results, such that the number of Iba1+ cells was not altered in the DG of EE rats compared to controls and the estimated hippocampal volumes were similar between housing conditions. However, the average Iba1+ cell volume was significantly reduced in EE rats, indicating a baseline reduction in soma size as a result of EE. This change in cell volume may be correlated to the attenuated cytokine and chemokine response we observed in the first enrichment study, such that smaller quiescent microglia respond less strongly to peripheral infection. Recent evidence indicates that ASC, a protein commonly associated with inflammasome complexes may also have an important role in cytoskeleton reorganization (315). Inflammasomes are intracellular protein complexes that assemble in response to a pathogenic signal and are key regulators of the innate immune response. ASC protein may be part of the mechanism linking the smaller cell size to reduced reactivity to peripheral challenge. Furthermore, the increased CD200R expression in EE rats from our previous enrichment study may be the cause of decreased microglial soma size by increasing microglial receptiveness to inhibitory neural signals via CD200.

While astrocyte (GFAP+) cell number was not altered by EE, it is important to note that GFAP is expressed by other cell types in the brain in addition to astrocytes and does not label entire cells (i.e., both somas and processes) when used as an immunohistochemical marker. Furthermore, counting GFAP+ cells does not address

changes in GFAP on a per-cell basis, such that the increase in antigen density that we previously measured may likely stem from increased GFAP expression per cell rather than an increase in cell numbers. It is possible that changes in GFAP might be evidence of alterations to many cell types within the DG rather than a change in astrocytes *per se*. Other methodologies, such as Western blot for GFAP or ALDH1l1 protein within the hippocampus, may better elucidate the changes in astrocytes that we believe are occurring during EE. Laser capture of the DG followed by one of those methods may be yet another, more accurate way to assess the effects of EE on the glia in the neurogenic niche. Unlike GFAP staining, the Iba1 antibody stain fills somas and processes on microglia. The distinction between smaller somas noted in this study and increased glial antigen density in the former study may be accounted for by Iba1 density on microglial processes, which were not analyzed here. Analyses of these Iba1+ cells likely accurately reflect the effects of EE at baseline prior to immune challenge.

Rather than a peripheral *in vivo* challenge that we performed in our earlier study, we instead isolated microglia (CD11b+ cells) and treated them *ex vivo* with lipopolysaccharide to assess the effects of EE on microglia alone. The cytokine and chemokine response of microglia to LPS in culture is not different between housing groups. Both groups had robust upregulation of cytokine and chemokine secretion. However, the response of hippocampal microglia in culture was significantly lower than microglia from the adjacent parietal cortex for several cytokines and chemokines,

indicating that the hippocampal microglia may be slightly less reactive overall. These data provide evidence that the hippocampus is distinct from other cortical areas, regardless of the influence of EE on microglia reactivity. The remaining hippocampal cells (CD11- population) had similar expression of fractalkine (CX3CL1) and CD200, replicating the previous study and indicating that changes in microglial reactivity are not caused by alterations in these inhibitory proteins from neurons.

Nevertheless, the results from the *ex vivo* challenge must be interpreted with some caution. The isolation process, though rapid, is capable of activating microglia, which can be seen in the detectable cytokine levels in the Media condition. The media used in this study also contained serum (FBS), a substance that microglia *in vivo* only encounter during grave injury or insult. While the serum in the media improved cell survival (data not shown), it may account for the increased baseline readings in the Media conditions as well as for the lack of group difference resulting from housing condition. Furthermore, microglia *in vivo* do not interact directly with LPS that is administered peripherally, as it does not cross the blood-brain barrier (BBB); thus, the response seen to LPS in culture may be a reaction that would be attenuated *in vivo* by the BBB and/or the presence of other cell types.

Taken together, these data indicate that the effects of environmental enrichment on microglia require the presence of and interaction among all cell types within the hippocampus. Without neurons and astrocytes, microglia from EE rats are equally

reactive to an immune challenge as microglia from controls. Thus, the modulation on microglial reactivity is likely interactions among the cells types of the entire microenvironment of the DG rather than alterations driven by EE that occur in microglia independently of others cells. The effects we observed in our previous study may be the consequence of interactions between microglia and other neural cells as well as growth factors and other changes in signaling that result from EE. Notably, hippocampal microglia are still distinct from adjacent cortical microglia, even when stimulated *ex vivo*, indicating a baseline regional difference that may be augmented by EE's effects on the hippocampus. The specific effects on astrocytes during EE remain to be fully explored, either using improved counting methods or other protein analyses. The profound effects of EE on glia and immune signaling with the hippocampus are just beginning to be explored and these data provide a greater understanding of the influence of enrichment on glial phenotype.

6. Summary and Conclusions

Animals rely on their hippocampus for encoding memories and navigating throughout their environments. The hippocampus itself is a unique cortical structure that is dynamic and plastic. It is one of only two regions known to continue producing and integrating new cells into its network through neurogenesis and is well-characterized as a region capable of molecular, cellular and network alterations that directly affect learning and memory. In addition to its flexibility, the hippocampus is vulnerable to disruptions both inside and outside of the CNS. The juxtaposition of these two traits – vulnerability and plasticity – may have similar underlying mechanisms. As recent research has shown, signaling by immune molecules and microglia modulate both hippocampal plasticity and vulnerability, and conversely, changes in hippocampal plasticity may alter immune signaling within the CNS.

The first goal of this dissertation research was to assess the roles of microglia and immune signaling molecules within the CNS in a model of hippocampal vulnerability. Neonatal infection occurs during the critical period of early postnatal brain development and microglial proliferation in the CNS. **Chapter 2** (Experiment 1) sought to define the molecular mechanism and cellular source of the fear conditioning memory impairment that occurs as a consequence of early-life infection. The results indicate that neonatally infected rats given LPS as adults ("two-hit" rats) have exaggerated interleukin-1β expression within the hippocampus during learning. These data demonstrate that

microglia (CD11b+ cells) are the primary source of that exaggerated IL-1β. Isolated microglia from neonatally infected rats are more reactive at baseline and following *ex vivo* stimulation with LPS. Furthermore, blocking glial reactivity with minocycline, either following LPS injection or following learning, eliminates the learning impairment in "two-hit" rats. The impairment requires "unmasking" by the second immune challenge in adulthood; however, this experiment showed that microglia in neonatally-infected rats are more reactive to external challenges and to learning events.

The second goal of this dissertation research, in **Chapter 3** (Experiment 2), was to further elucidate the effects of neonatal infection on hippocampal-dependent learning and to determine if neural activation within the hippocampus is affected by neonatal *E. coli* infection during learning. Acquisition of a platform location during a Morris Water Maze task was not affected by neonatal infection during minimal or extensive training. Neonatal infection did, however, lend an advantage during memory testing after minimal training, such that neonatally infected rats had better memory for the platform location 48HR later. Interestingly, this advantage disappeared after reversal training and neonatally infected rats were significantly impaired in their memory for the reversal platform. Thus, the greater precision after minimal training may be coupled to a lack of flexibility, such that a better memory for one location (i.e., the original platform) may interfere in the formation of the memory for another location (i.e., the reversal platform). The improved memory following minimal training was correlated with decreased Arc

protein expression within the dentate gyrus of the hippocampus. The reduction in Arc protein in the DG may be evidence of smaller neuronal network activation by the learned location and a possible correlation of that network to improved memory. During the reversal memory test, there were no differences in Arc protein expression in the DG between controls and neonatally infected rats, indicating that the hippocampus may not be the neural substrate most important for behavioral reversal. Indeed, the prefrontal cortex has been implicated in several types of reversal learning (274-277) and requires further study.

The results from the model of neonatal infection and its resulting hippocampal vulnerability led to the third goal of this dissertation research, which was to test the hypothesis that environmental enrichment, a manipulation known to increase hippocampal plasticity in several ways, can reduce or eliminate the vulnerability of the hippocampus to an immune challenge. In **Chapter 4** (Experiment 3), the results indicate that environmental enrichment has a dramatic effect on glial antigen expression (i.e., GFAP on astrocytes and Iba1 on microglia) and that EE significantly attenuates the pro-inflammatory response within the hippocampus to an LPS challenge. In response to the immune challenge, environmentally enriched rats had lower mRNA expression of IL-1β, TNFα and members of its signaling family, and several chemokines within the hippocampus, and notably, the response in the adjacent parietal cortex was not different between housing groups to LPS. Alterations in gene transcription were specific to the

hippocampus, and changes to glial antigen expression were confined to the neurogenic niche of the DG within the hippocampus. Further work with environmental enrichment (**Chapter 5**; Experiment 4) assessed the specific effects of EE on microglial reactivity as well as specific changes in glial cell number or morphology that contributed to increased glial antigen density measurements in **Chapter 4.** The results show that numbers of astrocytes and microglia are not altered within the DG, but microglia volume is reduced in response to EE. The reduction in microglia volume at baseline may correlate with the attenuated pro-inflammatory response seen following an *in vivo* immune challenge. However, isolated microglia that are stimulated *ex vivo* have the same robust response to LPS, regardless of housing condition. Hippocampal microglia, however, have a lower response to LPS than microglia isolated from the parietal cortex, indicating a possible baseline difference in microglial reactivity between these two regions. Notably, the microglial phenotype we observed *ex vivo* following enrichment does not replicate the reactivity observed *in vivo* after EE and an immune challenge. In the neonatal infection model, the *ex vivo* phenotype mirrored the *in vivo* phenotype with greater microglial reactivity observed in both experiments. Neonatal programming by infection during a critical period of brain and microglial development has a profound and enduring effect on hippocampal microglia phenotype, even in isolation from other cells, while enrichment has a robust effect on *in vivo* responses only. The manipulation of enrichment during adulthood may contribute to its potentially transient effects on the

inflammatory response within the hippocampus. Neonatal infection, however, can permanently program microglia well into adulthood and old age. These results indicate that it is likely the neurogenic niche of the DG and the microenvironment in which the microglia reside that determines their morphology and reactivity to immune challenge.

Taken together, the experiments in this dissertation have provided evidence that alterations in microglia reactivity and immune signaling, especially early in life, have dramatic effects on hippocampal-dependent learning and memory and that changes in hippocampal plasticity have significant effects on microglia reactivity and immune signaling within that region. The connections between these phenomena are just beginning to be elucidated, and these experiments provide evidence that microglia are critical for hippocampal function and that changes in plasticity have profound impacts on microglia, even in adulthood. Cytokines and chemokines are central molecules in hippocampal risk and resilience, and these molecules can no longer be considered solely immune signals, but perhaps neuromodulators as well. Specific relationships between cytokine signaling and neurotransmitters are as yet minimally explored and in the future, it will be important to consider how cytokines and chemokines influence neural plasticity at the network level. The broader implications of this work indicate that the hippocampus remains a critical neural substrate in understanding the complex relationships between the immune system and the CNS. Hippocampal-dependent neural function may be immune-dependent as well, and neuromodulation by immune

molecules may occur primarily in the hippocampus as a response to the region's remarkable plasticity.

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Biography

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